



Endocrine disrupters affecting male rat reproductive development - focus on phthalates and the fetal testis

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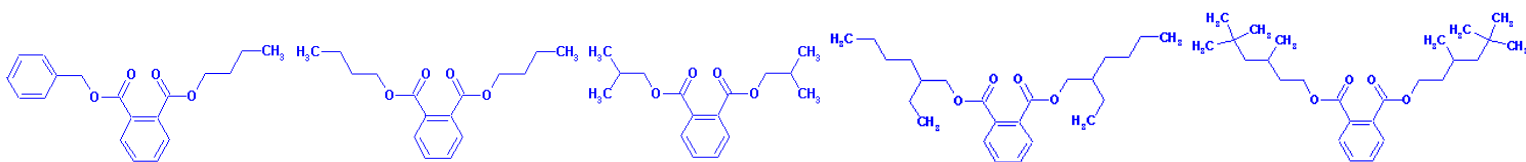
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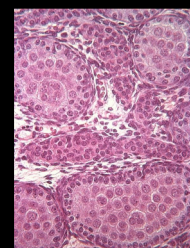
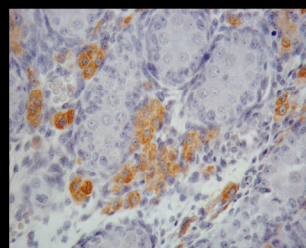
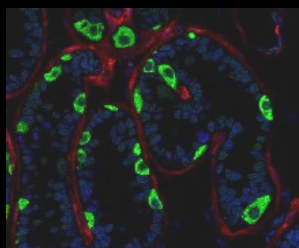
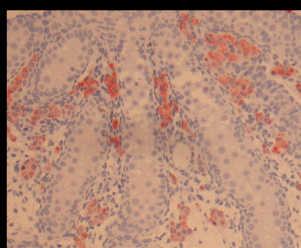
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Endocrine disruptors affecting male rat reproductive development

– Focus on phthalates and the fetal testis

PhD thesis by Julie Boberg



Preface

This PhD project was carried out at the National Food Institute at the Technical University of Denmark in cooperation with Roskilde University.

Numerous persons have participated in the work for this thesis. Among others, Ulla Baroudy, Vibeke Kjær, Birgitte Møller Plesning, Heidi Letting and Morten Andreasen are thanked for their excellent technical assistance and for always being helpful and patient. Majken Dalgaard and Rie Vinggaard have provided excellent supervision and inspiration - thank you very much for following my project to the end even though you are now both engaged elsewhere. Also thanks to Ole Vang, my supervisor at Roskilde University. Additionally, the members of the “repro- & hormone group” at the National Food Institute have contributed to the project with many good discussions and cooperation with the animal studies. Thanks to you and to everybody at the National Food Institute (particularly the Section of Pathology) who have contributed to a great working environment.

I have been fortunate to be able to visit two other labs during my PhD study to learn new methods and experience working in other labs. I would like to thank Richard Sharpe and his “Team Testis” at the MRC Human Reproductive Science Unit, Edinburgh, for your warm welcome and great helpfulness during my stay. Susanne Mandrup and Michael Børgesen are also thanked for welcoming me at the University of Southern Denmark and helping me set up the PPAR reporter gene assays.

The studies were supported by grants from the EU (EDEN project, QLK4-CT-2002-0603), the Directorate for Food, Fisheries and Agro Business, Denmark (FØSI 00-11), the Nordic Council of Ministers and the Danish Medical Council (grants no 22-03-0198 and 2107-04-0006).

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In vitro study on PPAR α and PPAR γ transactivation by environmental contaminants

Summary

BACKGROUND: Testosterone is vital to the development of normal male characteristics and function. However, chemicals present in foods and products in everyday use can impair the production of testosterone in experimental animals. Studies in Danish men have shown declining sperm production over several decades, and as environmental factors are suspected of being part of the cause, research in the link between chemical exposure and reproductive function is necessary. Certain phthalates, which are abundantly used in PVC and other materials such as paints and glue, are known to reduce testosterone production in fetal male rats leading to malformations of the reproductive system and impaired reproductive function later in life.

AIMS AND METHODS: This PhD project investigated prenatal exposure of male rats to phthalates and other chemicals suspected of affecting fetal testosterone production in order to gain more knowledge on these chemicals and thereby improve risk assessment. Several rat studies were performed using similar study designs: Pregnant Wistar rats were gavaged with test compounds from gestation day (GD) 7 to GD 21, when their male offspring was examined and compared to controls. In a few studies, other dams gave birth and were dosed until postnatal day 17, and male offspring was examined at various ages thereafter.

Diethylhexyl phthalate (DEHP) affects testicular development and function in rats and is increasingly being substituted with alternative plasticizers in consumer products. We investigated whether DEHP-like fetal testicular effects could be induced with diisononyl phthalate (DINP) or diisobutyl phthalate (DIBP), which are increasingly being used as substitutes for DEHP and other toxic phthalates. Furthermore, effects of different mixtures were examined: a) a mixture of DEHP and DINP, and b) a mixture of DEHP and another plasticizer, diethylhexyl adipate (DEHA), having a common metabolite. Additionally, the hormonal and histopathological effects of perinatal DEHP exposure of male rats were examined with focus on how the effects progress during the lifespan of the rat. Studies on testicular gene expression and immunohistochemistry were performed to elaborate on the mechanisms behind the reproductive effects of phthalates as well as the fungicide prochloraz.

Based on a hypothesis on the possible involvement of peroxisome proliferators-activated receptors (PPARs) in the downregulation of testosterone production by phthalates and other chemicals, a reporter gene assay was developed for screening of chemicals for interaction with PPAR α and PPAR γ . Additionally, PPAR-activating chemicals were tested for effects on testosterone production in fetal rat studies.

RESULTS AND DISCUSSION: DINP and DIBP reduced fetal testicular testosterone production and – content and affected fetal testicular histopathology similarly to the known reproductive toxicant DEHP, pointing to a similar adverse effect pattern for these phthalates. Combined exposure to DEHP and DINP induced accumulated effects compared to the effects seen with each compound alone.

The studies have elaborated on the temporal development of the testicular histopathological effects of DEHP, supporting the findings described for di-n-butyl phthalate (DBP) by other investigators. These findings include effects on Leydig cells, gonocytes and Sertoli cells in fetal testes. Later in life, some testes degenerated while most regenerated. However, dysgenetic areas were present in otherwise normal testes of

DEHP-exposed young and adult rats. No modulating effects of DEHA on the reproductive effects of DEHP were observed.

The testosterone-reducing effect of DEHP and DIBP is related to reduced levels of factors related to steroid synthesis. Protein levels of cytochrome P450 side chain cleavage (P450_{scc}), steroidogenic acute regulatory protein (StAR), cytochrome P450c17 (CYP17) and PPAR γ were less abundant in testes from phthalate-exposed fetuses as evaluated by immunohistochemistry. Correspondingly, mRNA levels of genes coding for these factors as well as scavenger receptor B-1 (SR-B1), steroidogenic factor-1 (SF1), and PPAR α were reduced in testes of phthalate-exposed fetal rats. Based on literature studies and indications from in vivo studies, it is suggested that the phthalate mechanism of action may involve the nuclear receptors SF1, PPAR γ and PPAR α , although further studies are needed.

Immunohistochemical studies on expression levels of factors involved in steroid synthesis elaborated on the mode of action of prochloraz. Prochloraz-exposed male fetuses had reduced testosterone synthesis, but no differences in the immunostaining intensity for P450_{scc} or StAR in Leydig cells. On the other hand, increased immunostaining intensity for CYP17 was seen in Leydig cells of prochloraz-exposed fetuses, emphasizing the importance of CYP17 in the mode of action of this compound. Additionally, a possible role of the enzyme 17 β -hydroxysteroid dehydrogenase (17 β HSD) type 10 in the mechanism of action of prochloraz was suggested due to reduced expression of 17 β HSD type 10 in Leydig cells of prochloraz-exposed fetuses. It is not clear whether this is a cause or an effect of the low testosterone production in prochloraz-exposed males.

The widely used preservatives butylparaben and propylparaben were shown to be weak agonists of PPAR α and PPAR γ in vitro. Further studies may reveal whether the interaction of parabens with PPARs can interfere with normal physiological processes in testes or other tissues. If these receptors are indeed involved in the testosterone-reducing effects of certain chemicals, reporter gene assays for PPAR α and PPAR γ such as those employed in the current studies may be valuable tools for screening chemicals for similar modes of action as phthalates, and may thus reveal other chemicals with potentially harmful effects.

CONCLUSIONS AND PERSPECTIVES: DINP and DIBP had similar effects on fetal rat testicular development and function as DEHP, and there is thus a reason for concern for the use of DINP and DIBP as substitutes for the reproductive toxicants DEHP or DBP. Furthermore, the observation of an accumulating effect when exposing rats to a mixture of DEHP and DINP indicates an increased risk in case of exposure to mixtures of chemicals with similar effects. Therefore, risk assessment should take into account the collective contribution of exposure to several reproductive toxic phthalates.

New insights have been made regarding phthalate and prochloraz mechanisms of action. It is not evident whether exposure to phthalates or other compounds interfering with fetal testosterone production or androgen function contributes to the testicular dysgenesis syndrome observed in humans. There appears to be a reason for concern that several chemicals have antiandrogenic effects and that the increasing incidence of male reproductive dysfunction may be due to combined exposure to antiandrogens. It is therefore highly relevant to be able to detect chemicals with antiandrogenic effects by use of adequate regulatory test guidelines and subsequently to be able to reduce human exposure to antiandrogens by regulatory initiatives.

Dansk resumé

BAGGRUND: Testosteron er vigtig for reguleringen af den mandlige udvikling. I de senere år har det vist sig, at kemikalier i fødevarer og forbrugerprodukter kan virke hormonforstyrrende ved bl.a. at hæmme dannelsen af testosteron hos forsøgsdyr. Samtidig har undersøgelser vist, at sædkvaliteten hos danske mænd er faldende og forekomsten af misdannelser i reproduktionssystemet stigende, og en sammenhæng med hormonforstyrrende stoffer er foreslået. For eksempel er visse phthalater, som anvendes i blandt andet PVC, lim og maling, mistænkt for at virke hormonforstyrrende hos mennesker, idet disse phthalater kan hæmme testosteronproduktionen hos hanrotter i fostertilstanden og medføre misdannelser samt nedsætte reproduktionsevnen senere i livet.

FORMÅL OG METODER: I dette PhD-projekt er foretaget en række studier af hanrotter, der i fostertilstanden er eksponeret for phthalater og andre forurenende kemikalier mistænkt for at påvirke testosteronproduktionen. Øget viden om disse stoffer og mekanismerne bag deres skadelige effekter ønskes for at kunne forbedre risikovurderingen. En række rottestudier er udført med udgangspunkt i et fælles forsøgsdesign: Drægtige Wistar rotter er oralt doseret med mulige hormonforstyrrende stoffer fra gestationsdag (GD) 7 til 21, hvor deres hanunger blev undersøgt og sammenlignet med ikke-doserede dyr. I enkelte studier er doseringen fortsat til dag 17 efter fødslen, og hanungerne er undersøgt i forskellige aldersgrupper.

Diethylhexyl phthalat (DEHP) påvirker testiklernes udvikling og funktion hos rotter og bliver i stigende grad erstattet med andre plastblødgørere i forbrugerprodukter. Vi har derfor undersøgt om DEHP-lignende effekter ses ved eksponering med diisononyl phthalat (DINP) eller diisobutyl phthalat (DIBP), der anvendes som erstatning for bl.a. DEHP. Desuden undersøgtes effekterne af kombineret eksponering for to kemikalier: a) en kombination af DEHP og DINP og b) en kombination af DEHP med en anden plastblødgører, diethylhexyl adipat, DEHA, som har visse træk tilfælles med DEHP. Desuden blev det undersøgt, hvordan de histologiske og hormonelle forandringer forårsaget af DEHP udviklede sig igennem rottens levetid. Immunhistokemiske studier samt genekspressionsanalyser blev udført for at øge kendskabet til mekanismerne bag de reproduktionsskadelige effekter af phthalaterne samt af sprøjtegiften prochloraz.

Da peroxisom proliferator-aktiverede receptorer (PPARs) kan tænkes at være involveret i phthalaternes testosteron-hæmmende virkning, er et reporter-gen assay udviklet for at kunne screene kemikalier for deres evne til at aktivere eller hæmme PPARs. I forlængelse af denne hypotese blev PPAR- som PFOA, rosiglitazon og butylparaben undersøgt for mulig påvirkning af testosteronproduktionen hos rottefostre.

RESULTATER OG DISKUSSION: DINP og DIBP havde samme virkning som DEHP, idet de reducerede testosteronproduktionen og forårsagede histopatologiske forandringer i testiklerne hos doserede rottefostre. Disse fund hos rottefostre tyder på, at DINP og DIBP kan have samme skadelige effekter som DEHP også senere i livet og derfor vil være uegnede som erstatninger for DEHP. Desuden medførte dosering med en kombination af DEHP og DINP en samlet effekt som var mere markant end effekten af de enkelte stoffer.

Undersøgelserne af den tidsmæssige udvikling i de histologiske forandringer som følge af DEHP eksponering viste ligheder med observationer i forsøg med di-n-butyl phthalat beskrevet i litteraturen. Hos

DEHP-eksponerede fostre sås forandringer af Leydig celler, Sertoli celler og kønsceller i næsten alle testikler, mens der hos voksne rotter snarere sås et alt-eller-intet respons, idet visse testikler var degenererede og uden sædceller, mens de fleste forekom normale. Endvidere sås dysgene områder i testiklerne hos unge og voksne DEHP-doserede rotter. Eksponering for en kombination af DEHP og DEHA tydede ikke på at DEHA modulerede effekten af DEHP.

Den lave testosteronproduktion hos phthalat-doserede rotter er relateret til en nedregulering af gener og proteiner i steroidsyntesen. Immunhistokemiske studier påviste lavere proteinniveauer af cytochrome P450 side chain cleavage (P450scc), steroidogenic acute regulatory protein (StAR), cytochrome P450c17 (CYP17) og PPAR γ i Leydig celler hos phthalat-eksponerede fostre. Ligeledes var mRNA for disse faktorer såvel som for scavenger receptor B-1 (SR-B1), steroidogenic factor-1 (SF1) og PPAR α reduceret i testikler fra phthalat-eksponerede dyr. Forandringerne i protein- eller genekspression for SF1, PPAR γ og PPAR α understøtter hypotesen om, at disse kernereceptorer er involveret i phthalaternes virkningsmekanisme, men yderligere studier ønskes for at belyse dette.

Virkningsmekanismen for prochloraz er belyst ved hjælp af immunhistokemiske studier, der påviste øget niveau af CYP17 i Leydig celler i testes hos prochloraz-doserede fostre i forhold til kontroldyr. Desuden sås et reduceret proteinniveau af enzymet 17 β -hydroxysteroid dehydrogenase (17 β HSD) type 10, som dermed kan tiltænkes en rolle i prochloraz' virkningsmekanisme, selvom det endnu ikke er afklaret, hvorvidt det lave proteinniveau er en årsag til eller en følge af det lave testosteronniveau hos de prochloraz-doserede fostre.

Reportergen assays blev anvendt til at undersøge transaktivering af PPAR γ og PPAR α . Butylparaben og propylparaben viste svag agonisme til begge receptorer, men det er endnu uafklaret om denne interaktion kan påvirke de normale fysiologiske processer i testes eller andre væv. Hvis disse receptorer er involveret i den testosteron-hæmmende virkning af phthalater og andre kemikalier, vil reportergen assays som de her anvendte være et værdifuldt værktøj til at screene en mængde kemikalier for, om de har virkningsmekanismer, der ligner phthalaternes, og dermed muligvis er sundhedsskadelige.

KONKLUSIONER OG PERSPEKTIVER: Rottestudier viste at DINP og DIBP forstyrrer testiklernes udvikling ligesom DEHP, og disse stoffer bør derfor ikke anvendes som erstatning for de reproduktionsskadelige phthalater. Eksponering for en kombination af DEHP og DINP medførte mere markante hormonforandringer end observeret for de enkelte stoffer. Risikovurderingen bør derfor tage hensyn til menneskets samlede eksponering for mange forskellige phthalater med samme effekter.

Studierne har desuden belyst nye aspekter af mekanismerne bag den lave testosteronproduktion i fostertestikler efter eksponering for phthalater eller prochloraz. Det er næppe sandsynligt, at eksponering for phthalater eller andre enkeltstoffer med antiandrogen virkning er direkte årsag til den øgede forekomst af misdannelser i reproduktionssystemet og nedsat sædkvalitet hos mennesker. Der synes dog at være grund til bekymring for, at mennesket er eksponeret for en del kemikalier med antiandrogen virkning, og at den samlede antiandrogene påvirkning kan medføre hormonforstyrrelser og skader på mænds reproduktionssystem. Det er derfor yderst relevant at udvikle metoder til at detektere kemikalier med antiandrogene effekter, samt efterfølgende at gøre det muligt at reducere den humane eksponering for antiandrogener ved hjælp af regulatoriske tiltag.

List of published papers

- I. Borch J, Ladefoged O, Hass U, Vinggaard AM. Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats. *Reprod Toxicol*. 2004 Jan-Feb;18(1):53-61.
- II. Borch J, Dalgaard M, Ladefoged O. Early testicular effects in rats perinatally exposed to DEHP in combination with DEHA--apoptosis assessment and immunohistochemical studies. *Reprod Toxicol*. 2005 Mar-Apr;19(4):517-25.
- III. Jarfelt K, Dalgaard M, Hass U, Borch J, Jacobsen H, Ladefoged O. Antiandrogenic effects in male rats perinatally exposed to a mixture of di(2-ethylhexyl) phthalate and di(2-ethylhexyl) adipate. *Reprod Toxicol*. 2005 Mar-Apr;19(4):505-15
- IV. Borch J, Metzдорff SB, Vinggaard AM, Brokken L, Dalgaard M. Mechanisms underlying the antiandrogenic effects of diethylhexyl phthalate in fetal rat testis. *Toxicology*. 2006 Jun 1;223(1-2):144-55.
- V. Borch J, Axelstad M, Vinggaard AM, Dalgaard M. Diisobutyl phthalate has comparable antiandrogenic effects to di-n-butyl phthalate in fetal rat testis. *Toxicol Lett*. 2006 Jun 1;163(3):183-90.
- VI. Laier P, Metzдорff SB, Borch J, Hagen ML, Hass U, Christiansen S, Axelstad M, Kledal T, Dalgaard M, McKinnell C, Brokken LJ, Vinggaard AM. Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz. *Toxicol Appl Pharmacol*. 2006 Jun 1;213(2):160-71.

Please note that all papers were published using my maiden name Borch.

Abbreviations

17 β HSD	17 β -hydroxysteroid dehydrogenase	InsI3	insulin-like receptor-3
2-EH	2-ethylhexanol	LH	luteinizing hormone
3 β HSD	3 β -hydroxysteroid dehydrogenase	LOAEL	lowest observed adverse effect level
AGD	anogenital distance	MBP	monobutyl phthalate
AR	androgen receptor	MEHA	mono(2-ethylhexyl) adipate
BBP	benzylbutyl phthalate	MEHP	mono(2-ethylhexyl) phthalate
CYP17	cytochrome P450c17	MLTC-1	mouse Leydig tumor cells
DBP	di-n-butyl phthalate	NOAEL	no observed adverse effect level
DEHA	di(2-ethylhexyl) adipate	NS	not statistically significant
DEHP	di(2-ethylhexyl) phthalate	P450scc	cytochrome P450 side chain cleavage
DEP	diethyl phthalate	PBR	peripheral benzodiazepine receptor
DIBP	diisobutyl phthalate	PFOA	perfluoro octanoate
DIDP	diisodecyl phthalate	PND	postnatal day
DINP	diisononyl phthalate	PPAR	peroxisome proliferator-activated receptor
DMP	dimethyl phthalate	SF1	steroidogenic factor-1
DnOP	di-n-octyl phthalate	SR-B1	scavenger receptor B-1
DOTP	dioctyl tere phthalate	SREBP	sterol regulatory element binding proteins
DPP	dipentyl phthalate	StAR	steroidogenic acute regulatory protein
GD	gestation day	TDI	tolerable daily intake
hCG	human chorionic gonadotropin		

1 Introduction

Testosterone is vital to the development of normal male characteristics and function. However, chemicals present in foods and products in everyday use can impair the production of testosterone in experimental animals. Studies in Danish men have shown declining sperm production over several decades, and as environmental factors are suspected of being part of the cause, research in the link between chemical exposure and reproductive function is necessary. Of special interest are the phthalates, which are abundantly used in PVC and other materials such as paints and glue. Certain phthalates are known to reduce testosterone production in fetal male rats leading to impaired reproductive function later in life.

This PhD project aimed to investigate prenatal exposure of male rats to phthalates and other chemicals suspected of affecting fetal testosterone production. This is important in order to gain more knowledge on these chemicals and thereby improve risk assessment. Another perspective is that knowledge on the mechanism of action of phthalates may be applied in the development of in vitro methods to screen other environmental compounds for similar effects. Additionally, mechanistic knowledge can be a key to understanding whether the effects are relevant to humans.

This project mainly focused on phthalate effects in vivo. The hormonal and histopathological effects were examined in male rats perinatally exposed to phthalates and other compounds (prochloraz, butylparaben, perfluorooctanoate, rosiglitazone) suspected of disrupting reproductive development. The phthalate studies examined mechanisms of action, dose-response relationships and effects of mixtures. Additionally, a hypothesis on the possible involvement of peroxisome proliferators-activated receptors (PPARs) in the testosterone-reducing effect of phthalates encouraged the development and application of reporter gene assays for screening of chemicals for interactions with PPAR α and PPAR γ .

2 Background

The following sections describe the hypothesis of environmental influences on male reproductive function. As this area has received much attention during the last decade, focus will mainly be on studies on phthalate effects in order to form a background for the performed studies.

2.1 Declining male reproductive function and endocrine disrupters

The number of children born per person in the industrialized and the developmental world has been declining within the last decades (reviewed in ^{1,2}). Obviously, the majority of this decline can be related to changing family patterns and new possibilities in birth control. In theory, the reduced fertility rate in industrialized countries may not be important on a global level, as many developing countries still have rapidly growing populations. However, social factors may not be the only explanation. Studies have indicated that incidences of disorders in the male reproductive system have been rising within the last 50 years in Denmark ³. If these trends are due to environmental and lifestyle influences on reproductive health, the “westernization” of developing countries may be associated with global consequences for the human population in the future.

Testis cancer and malformations of the external male genitalia are now very frequent in Danish young men among whom 15 per 100,000 develop testicular cancer and up to 20 % have low semen quality in the infertile range ^{4,5}. Incidences of hypospadias and cryptorchidism have been increasing since the 1960's in many countries ³. In Denmark, 5 % of boys exhibit some degree of hypospadias, 9% are born with cryptorchidism and 2% are cryptorchid at the age of 3 months ^{6,7}. As cryptorchidism is a risk factor for reduced semen quality and development of testis cancer, these statistics seem alarming.

The testicular dysgenesis syndrome has recently been suggested as a pattern of human reproductive disorders presumed to have a common origin ⁸. Fig. 1 illustrates this hypothesis, i.e. how reduced semen quality, testis cancer, hypospadias and testicular maldescent may be related to testicular dysgenesis interfering with Sertoli and Leydig cell function. In some cases, testicular dysgenesis is associated with genetic defects, while the hypothesis that this syndrome may be related to environmental contaminants (endocrine disrupting chemicals) has been investigated and debated intensely in recent years.

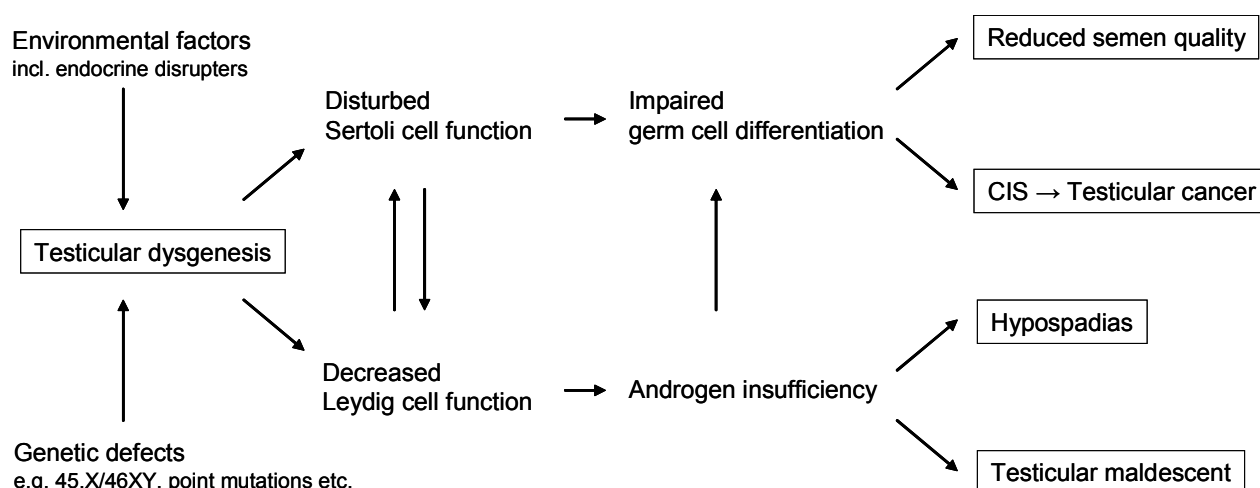


Fig. 1. Model of associations between components of the testicular dysgenesis syndrome. Genetic and environmental factors may contribute to testicular dysgenesis, i.e. disturbed development of Leydig, Sertoli and germ cells. Malfunction of these cell types may lead to reduced semen quality, testis cancer development, hypospadias and cryptorchidism. From Skakkebaek et al., 2001 ².

Animal studies confirm that endocrine disrupting chemicals are able to induce the testicular dysgenesis syndrome. In order to evaluate the plausibility of an environmental influence on the development of the testicular dysgenesis syndrome, information on human exposure to endocrine disrupting chemicals is important.

Historically, the effects of chemical exposure in humans have mainly been studied in work exposure settings. Studies have revealed effects of occupational exposures to metals, solvents and pesticides on human male reproduction (reviewed in ⁹). Recent studies in the normal population have aimed to correlate the reproductive development of infants to chemical exposure of the mother and child. A study on 100 children from the US showed associations between high urinary levels of certain phthalate metabolites and a low anogenital index, a marker of “masculinization” of male external genitalia ¹⁰. Additionally, a study on Danish and Finnish children indicated correlations between reproductive hormone levels in serum of 3 months old boys and levels of certain phthalate esters in their mothers’ breast milk ¹¹. Breast milk levels of some phthalates were found to be negatively associated with free testosterone levels and positively correlated with LH:testosterone levels in boys’ serum ¹¹. This points to poor function of the testosterone producing cells in boys exposed to high levels of phthalates. Further investigations are necessary in order to confirm whether these correlations are indeed signs of adverse effects of phthalates on male reproductive development.

2.2 Phthalates – use and toxicity

Phthalates are produced in very high volumes and are present in materials in everyday use. Although phthalates and their primary metabolites have a short half-life and thus may not accumulate in the body, the abundance of the chemicals leads to measurable levels in human blood and breast milk ¹¹. The main route of exposure is via food, as phthalates present in the environment end up in e.g. water, fish and plants ¹². Oral

exposure of young children via mouthing of toys containing phthalates is also considerable, although the use of certain phthalates in infant toys has recently been banned in the EU. Additionally, humans may be exposed via inhalation of dust from indoor air containing phthalates from building materials and consumer products or via dermal exposure caused by contact with e.g. clothes and gloves containing phthalates¹². In addition to the general abundance of phthalates in consumer products, medical products such as tubes and tablet coating can contain extremely high levels of phthalates.

Phthalates are present as diesters in materials, but after ingestion these are rapidly metabolized to their monoesters. Phthalate monoesters are absorbed in the gut and further modified by hydroxylation, oxidation and glucoronidation before urinary excretion^{13;14}. Phthalate monoesters are also transported across the placenta¹⁵ and can be measured in amniotic fluid of humans as well as phthalate-exposed rats^{13;16}.

It has been well documented that certain phthalates have adverse effects on male rat reproduction with particular risk in case of perinatal exposure. The adverse reproductive effects seen in adult rats after perinatal exposure is very similar for phthalate esters with side chain lengths of 4 to 6 carbons in the ortho-configurations (diethyl hexyl phthalate (DEHP), benzylbutyl phthalate (BBP), di-n-butyl phthalate (DBP) and diisononyl phthalate (DINP))¹⁷. In contrast, phthalates with shorter ester side chain lengths (diethyl phthalate (DEP) and dimethyl phthalate (DMP)) or a para-configuration (dioctyl tere phthalate (DOTP)) do not alter reproductive development¹⁷.

The first evidence that certain phthalates such as DEHP, DBP and BBP are testicular toxicants was reported around 1980¹⁸⁻²⁰. As these earliest studies were performed in prepubertal or adult rats, high doses were required to induce adverse effects. Studies performed in the 1990's revealed that the intrauterine period is more sensitive to phthalate exposure, and that exposure to these phthalates in late gestation can induce adverse reproductive effects at lower doses^{21;22}. However, it was not until 2005 that these three compounds were permanently banned for use in all toys and childcare products in the EU²³. DINP, DiDP (diisodecyl phthalate) and DnOP (di-n-octyl phthalate) were concomitantly banned for use in toys for children under 3 years of age²³. This was an extension of a temporary ban of these six phthalates in infant toys decided in 1999²⁴. As a result of the increasing awareness of the effects of these compounds, DEHP, DBP and BBP are currently being phased out in certain materials, while the industrial use of less well-investigated phthalates may be increasing²⁵.

Adult male rats exposed to the reproductive toxic phthalates during development have an increased incidence of hypospadias, cryptorchidism, testicular atrophy, reduced sperm counts and malformations or hypoplasia of epididymis, vasa deferentia, seminal vesicles and prostate^{17;26;27}. This pattern of effects is very similar to the human testicular dysgenesis syndrome, and causal relations between phthalate exposure and development of testicular dysgenesis have thus been suggested, but also other chemicals can induce similar types of effects in rats.

2.3 Mechanisms of antiandrogenic effects in male rats

Many different types of chemicals can affect male reproductive development in animal studies. Some of these chemicals can be categorized as having estrogen-like effects in tissues, while others rather appear to

disrupt the production or function of androgens and are called antiandrogens. The observed effects in male rat offspring perinatally exposed to antiandrogens include reduced weights of reproductive organs, increased incidences of cryptorchidism and malformations of external genitalia, alteration of hormone levels, and decreased sperm quality. Among the antiandrogenic chemicals, some are known to be antagonists to the androgen receptor (AR), while others inhibit 5 α -reductase, an enzyme converting testosterone to the metabolite dihydrotestosterone, which has strong affinity for the AR. Direct or indirect influences on steroid converting enzymes can also lead to reduced testosterone production.

Table 1 compares the antiandrogenic effects of various chemicals in perinatally exposed rats. Some act through inhibition of the AR and others inhibit fetal testosterone synthesis, but they all affect the androgen dependent development of male external genitalia. The anogenital distance (AGD) is longer in normal males than in females, and a reduced AGD is thus a sensitive marker for antiandrogenicity and is affected by all these chemicals. The pattern of malformations such as the degree of hypospadias or cryptorchidism and the epididymal agenesis or underdevelopment appears to differ between these compounds and may in part depend on other factors than androgens. For example, the development of cryptorchidism is also related to altered levels of Insulin-like factor 3 (InsI3).

Table 1. The listed chemicals induce antiandrogenic effects in rats. AR antagonism, reduced fetal testosterone production and/or reduced InsI3 levels lead to reduced anogenital distance and malformations such as hypospadias, cryptorchidism, and epididymal dysgenesis. Modified from Gray et al. (2006) ²⁸.

Chemical	AR effect	Fetal T synthesis	InsI3 mRNA level	AGD effect	Hypospadias	Cryptorchidism	Epididymal dysgenesis
Vinclozolin	↓	-	-	↓	+++	++	-
Procymidone	↓	-	-	↓	+++	+	-
p,p'-DDE	↓	?	?	↓	+	-	-
Linuron	↓	↓	-	↓	+	-	++
Prochloraz	↓	↓	-	↓	+++	-	-
DEHP	-	↓	↓	↓	++	+++	+++
BBP	-	↓	↓	↓	++	+++	++
DBP	-	↓	↓	↓	+	+	++

Among the AR antagonists are five pesticides, vinclozolin, procymidone, p,p'-DDE, linuron and prochloraz (Table 1). Some phthalates and at least two of the mentioned AR antagonists (linuron and prochloraz) also reduce fetal testosterone synthesis (Table 1). The fungicide prochloraz is an example of a compound that is both an AR antagonist and inhibits fetal testicular testosterone production ^{29;30}. In contrast, neither the phthalate diesters nor their primary monoester metabolites are strong AR antagonists despite similarities to other antiandrogens regarding their effects in vivo. However, recent studies indicate that secondary DEHP metabolites are able to antagonize the AR ³¹. As will be discussed in the following, it is well described that some phthalates reduce fetal testosterone production, but the underlying cause has not been determined.

Thus, several classes of chemicals have the potential to be involved in the observed increase in male reproductive disorders in the industrialized world. The level of exposure to each chemical or chemical group alone may not be sufficient to induce adverse effects in humans, but as humans are exposed to numerous

chemicals with various effects on the reproductive system, the combined effect of these chemicals may be a cause for concern.

2.4 Effects of perinatal phthalate exposure of rats

As mentioned, perinatal exposure to certain phthalates can induce hypospadias, cryptorchidism, testicular atrophy, reduced sperm counts and malformations of reproductive organs. These effects originate in fetal life, when testosterone production is reduced and the development of fetal testes is disrupted³². Fetal testes of rats exposed to DEHP or DBP show characteristic morphological changes not observed with any other chemicals than phthalates. Seminiferous chords appear larger with more gonocytes, which are centrally located and some are multinucleated. Leydig cells are small and aggregated into large clusters^{33;34}.

This abnormal morphology of the Leydig cells correlates with their low production of testosterone. Normally, testicular testosterone production peaks from gestation day (GD) 17 to PND 2, but this peak is absent in phthalate-exposed males³³⁻³⁵ (Fig. 2).

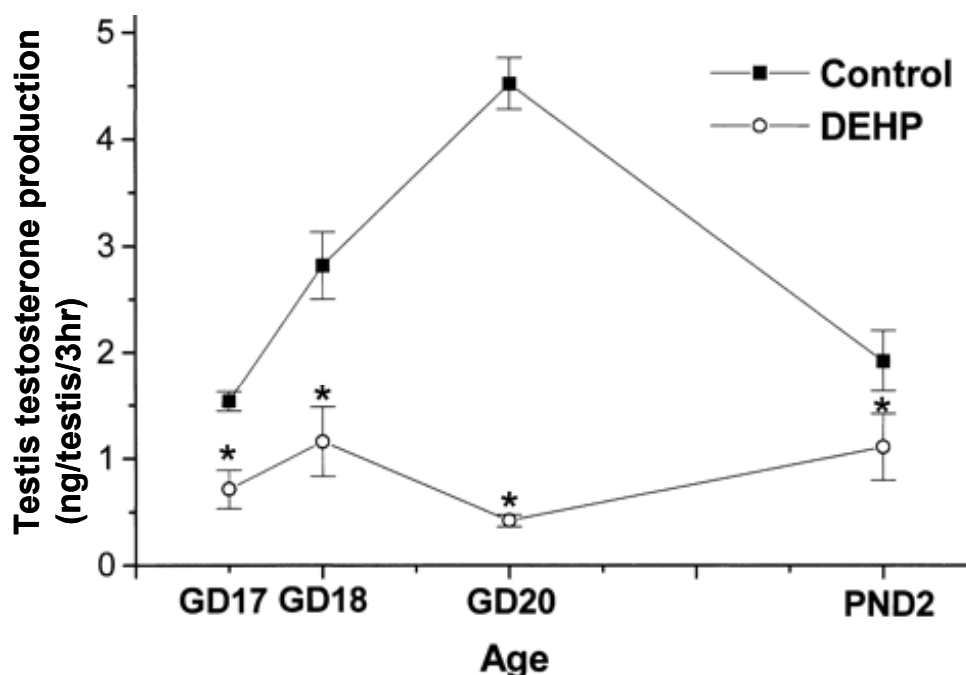


Fig. 2. Testicular testosterone production peaks around gestation day (GD) 20 in normal male rat fetuses, but this peak is not seen in DEHP-exposed rats. From Parks et al 2000³⁴.

The molecular origin of the testosterone-reducing effects of phthalates has been examined in several studies in recent years. In 2001, Shultz et al. described how fetal testicular expression of genes coding for factors involved in testosterone synthesis, fatty acid and cholesterol synthesis, cell death and proliferation was affected by DBP exposure from GD 12 to GD 21. They emphasized the down-regulation of scavenger receptor B-1 (SR-B1) and steroidogenic acute regulatory protein (StAR), which code for proteins transporting cholesterol across the Leydig cell membrane and the mitochondrial membranes, respectively³⁵. In addition, the gene expression of cytochrome P450 side chain cleavage (P450scc) and cytochrome P450c17

(CYP 17), which are important enzymatic steps in the conversion of cholesterol to testosterone, were down-regulated³⁵ (Fig. 3).

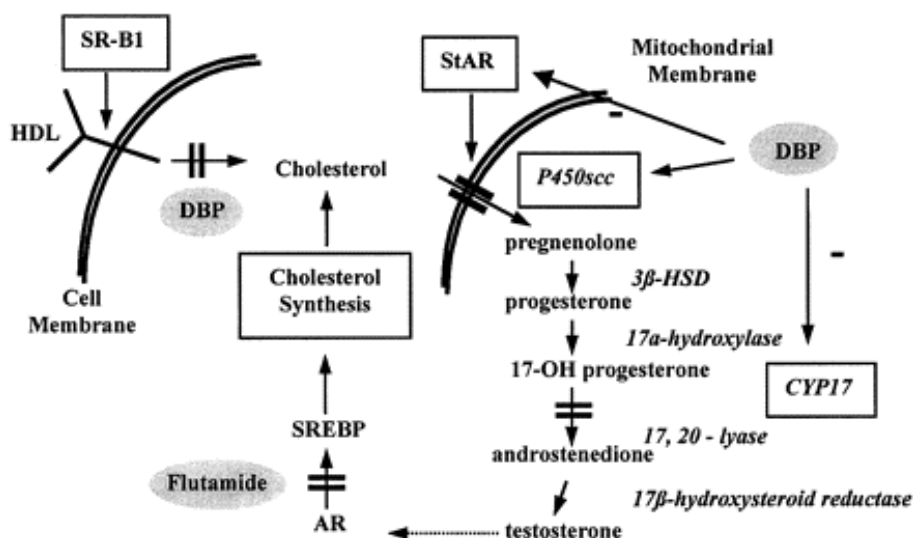


Fig. 3. Model of phthalate effects on factors involved in steroid synthesis. DBP affects the mRNA expression of *SR-B1*, *StAR*, *P450_{scc}* and *CYP17* genes in fetal testes thereby reducing testosterone levels. In contrast, androgen receptor antagonists such as flutamide rather acts by disrupting the effects of androgens in tissues, but may also reduce testosterone synthesis via the influence of AR on factors involved in cholesterol synthesis. From Shultz et al. (2001)³⁵.

As several steps in the steroidogenic pathway were affected by DBP, it appears likely that these effects are secondary and downstream to a common primary target still waiting to be identified. The transcription of genes coding for proteins involved in steroid synthesis are regulated by several ligand-activated nuclear receptors, which are activated by ligand binding and subsequently bind to response elements on their target genes. The androgen receptor (AR), steroidogenic factor 1 (SF1) and peroxisome proliferator activated receptors (PPARs) regulate some of the genes coding for proteins involved in steroid synthesis. SF1 regulates the transcription of several factors involved in steroid synthesis including SR-B1, StAR, P450_{scc}, 3 β -hydroxysteroid dehydrogenase (3 β HSD) and CYP17^{36,37}. AR regulates the activity of sterol regulatory element binding proteins (SREBPs), which regulate the transcription of genes for SR-B1, StAR and other enzymes involved in the maintenance of cholesterol homeostasis³⁸⁻⁴⁰. In addition to regulating the hepatic cholesterol homeostasis and thereby the substrate availability to steroid synthesis, PPARs regulate the transcription of SR-B1 and the cholesterol transporter peripheral benzodiazepine receptor (PBR), which cooperates with StAR in mitochondrial cholesterol uptake⁴¹. It is therefore relevant to examine whether these nuclear receptors interact with phthalates or other chemicals influencing fetal testosterone production. In vitro studies have not revealed substantial evidence for interaction between phthalate monoesters and AR or SF1^{31,42}, while it is well known that PPARs are activated by certain phthalates⁴³.

Corton and Lapinskas (2005) have reviewed the literature on the possible involvement of PPARs on the testicular effects of phthalates. They find some degree of correlation between the reproductive effects of

phthalate esters and the ability to transactivate PPAR α or PPAR γ in reporter gene assays⁴⁴. However, the authors find that the hypothesis has some weaknesses as some phthalates do not fit into this relationship, and they conclude that further studies are required to clarify PPAR-dependent and –independent events involved in the testicular toxicity of phthalates.

In addition to phthalates, industrial chemicals such as perfluorinated compounds and medical products such as fibrates and thiazolidinediones are also known to activate PPAR α or PPAR γ as well as affect steroid production in testes and ovaries of rats and humans in vitro and in vivo^{41;45-49}. Bearing this in mind, it is a plausible hypothesis that PPARs may be involved in the testicular toxicity of not only phthalates, but also other PPAR activating chemicals.

2.5 Other PPAR activating chemicals affecting testosterone levels

For several compounds there appears to be a link between PPAR activation and impaired testosterone production. Perfluorooctanoate (PFOA) and perfluorooctanesulfonate were previously used for surface treatment and emulsification, and PFOA is known to activate PPAR α in transactivation studies⁵⁰. Human blood levels of PFOA and similar compounds have increased since the 1970s, but due to their accumulation in the environment, some of these compounds have recently been phased out for certain applications by the manufacturer⁵¹. Ammonium PFOA exposure for 14 days reduced hCG-stimulated serum testosterone levels in adult male rats in a study by Cook et al (1992) and reduced testosterone levels in rat serum and testicular interstitial fluid in another study by Biegel et al (1995)^{45;46}.

Another compound linking PPAR activation and impaired testosterone production is the PPAR γ agonist rosiglitazone, a thiazolidinedione drug, which was found to reduce testosterone levels in normal men⁴⁹. Rosiglitazone is used for treatment of women with polycystic ovary syndrome and reduces hyperandrogenism in these patients^{52;53}. These observations indicate that it is plausible that testicular effects in rats exposed to PPAR activating environmental compounds may also be relevant to humans.

Also certain parabens, which are abundantly used in moisturizers and cosmetics, have been found to reduce testosterone production in rats. Exposure of young male rats to either butylparaben or propylparaben reduced serum testosterone levels and decreased sperm production^{54;55}, while a third study showed that butylparaben reduced sperm number in perinatally exposed rats⁵⁶. Some parabens are also known to be estrogenic in vitro and in vivo, and as parabens have been measured in human breast tumor samples, a causal relation to breast cancer has been speculated⁵⁷. No studies on the interaction of parabens with PPARs have yet been reported, but due to the potentially high levels of human exposure, it is relevant to examine their toxicological effects further.

The evidence for effects of PFOA and butylparaben on testosterone production in young or adult rats have lead to speculations that these compounds may also affect fetal testicular testosterone production as seen for certain phthalates. If testicular testosterone production is indeed affected by several types of chemicals at this critical time of reproductive development, this may be highly relevant for risk evaluation for these chemicals. Not only will their individual levels of effect need to be considered, but also the effect of several compounds with similar mechanisms of action may need to be taken into account.

2.6 Mixture effects and risk assessment

Evidently, humans are environmentally exposed to a mixture of various chemicals some of which may have endocrine activity, and it is conceivable that the overall effect of mixed exposure will result in more severe effects than observed for each chemical alone. Risk assessment of toxicological effects is currently based on determination of thresholds for effects of single compounds, i.e. the lowest observed adverse effect levels (LOAELs) and the no observed adverse effect levels (NOAELs). However, it is highly relevant to consider combination effects of compounds with similar modes of actions, but a useful approach needs to be developed before this can be incorporated in risk assessment.

Combination effects have been studied intensely in cell-based studies, which have shown that combined exposure to several endocrine disrupting chemicals with similar modes of actions leads to increased effects. Most often, these combined effects have been shown to be concentration-additive (dose-additive). In animal studies, chemicals often have more complex effects and the interpretation of combination studies may be difficult. For example, various enzymatic processes drive the uptake and metabolism of chemicals, and as two chemicals may inhibit the uptake or metabolism of each other, combined exposure to the chemicals may not show dose-addition despite similar effects on their molecular target, e.g. at receptor level.

DEHP is hydrolyzed in the gut to its monoester, mono(2-ethylhexyl) phthalate (MEHP), and 2-ethylhexanol (2-EH). The metabolite 2-EH is also produced by hydrolyzation of another plasticizer, di(2-ethylhexyl) adipate (DEHA), which is being used as a substitute for DEHP. If part of the reproductive effects of DEHP can be attributed to the metabolite 2-EH, exposure to DEHA may induce adverse effects comparable to those seen with DEHP. Additionally, it may be hypothesized that combined exposure to DEHA and DEHP leads to increased effects compared to the effects of DEHP alone.

3 Aims of the studies

The purpose of the performed studies was to improve the knowledge basis for risk assessment by examining the following main topics:

- Identification of which phthalate esters affect reproductive development in the male rat and examination of dose-response relationships and effects of different mixtures: a) a mixture of two phthalates, and b) a mixture of a phthalate (DEHP) and another plasticizer (DEHA) having a common metabolite
- Hormonal and histopathological effects of perinatal phthalate exposure of male rats – focus on how the effects progress during the lifespan of the rat
- Mechanistic studies on the effects of phthalates on reproductive development in male rats
- Mechanistic studies on the effects of prochloraz on reproductive development in male rats
- Evaluation of a reporter gene assay for screening of chemicals for interaction with PPAR α and PPAR γ
- Test of PPAR activating compounds for effects on testosterone production in fetal rat studies

Effects of different phthalate esters - dose-response and combination studies:

Studies on the effects of phthalates have often been made with doses of 500 to 1000 mg/kg bw per day of DEHP or DBP. Other phthalates have been studied less than e.g. DEHP, but are suspected of having similar adverse effects. As DINP and diisobutyl phthalate (DIBP) are increasingly used industrially as substitutes for DEHP and DBP, we have tested these two phthalates for effects on testosterone production and histopathology of fetal testes, as well as the effect of mixed exposure to DEHP and DINP administered together.

Additionally, a hypothesis that DEHA may modulate the effects of DEHP when these compounds are administered in combination was examined by comparing the effects of mixed exposure to DEHP and DEHA to the effects observed with DEHP alone.

Furthermore, we aimed to examine the fetal testicular effects of lower doses of DEHP.

Hormones and histology – temporal development:

The fetal period is generally believed to be particularly vulnerable to chemicals interfering with hormonal balances⁵⁸. We wished to examine how perinatal phthalate exposure would affect male rats in fetal life compared to prepuberty and adulthood by examining testicular histopathology, apoptosis levels and various immunohistochemical markers in different age groups of DEHP-exposed rats.

Mechanistic studies:

Reduced fetal testosterone production is considered to be a biologically plausible as a mode of action for phthalate-induced reproductive effects³². However, the events leading to reduced fetal testosterone

production are not evident. Most likely, the reduction of fetal testosterone production is a result of reduced expression of genes coding for proteins/enzymes involved in steroid synthesis. As mentioned, PPARs and SF1 regulate some of these genes, and interaction of these receptors with phthalate metabolites may be hypothesized of playing a role in the downregulation of steroidogenesis. In the current project, we examined whether PPAR α , PPAR γ or SF1 gene and/or protein expression in testes of fetal rats were affected by phthalates.

The antiandrogenic fungicide prochloraz also reduces fetal testosterone production, and by methods comparable to those applied in the phthalate studies we wished to examine the gene and protein expression of factors involved in steroid synthesis

Screening of chemicals for PPAR interaction:

By developing a reporter gene assay, we wished to be able to identify some PPAR activating chemicals. In line with the hypothesis of a role of PPARs in the testosterone-reducing effect of phthalates and other PPAR agonists, we wished to examine whether other chemicals with effects on testosterone synthesis also interact with PPARs. As certain parabens reduce testosterone levels in rats, these parabens were tested for interaction with PPAR α and PPAR γ in reporter gene assays.

Test of PPAR activators for effects on fetal testosterone production

We hypothesized that if the interaction of phthalates with PPARs is causally involved in the reduction of fetal testosterone production, then other PPAR ligands may have similar effects in fetal rats. In a similar setup to the phthalate studies, testicular testosterone production, histopathology, gene and protein expression was examined in rat fetuses exposed to the PPAR ligands PFOA and rosiglitazone. In the same study, a group of rats exposed to butylparaben was included.

4 Experimental setup

Detailed descriptions of in vivo studies can be found in the papers I to VI (appendix X). The in vivo and in vitro studies on PPAR agonists are described in appendix Y. As listed in Table 2, a number of rat studies were performed with comparable study designs. Pregnant rats were gavaged with single doses or mixtures of test compounds from gestation day (GD) 7 to 21 and male fetuses were examined at GD 21. In other studies, exposure continued from postnatal day (PND) 1 to 17 and pups were examined in prepuberty and adulthood.

The offspring were then examined with different methods:

- Pathological and immunohistochemical investigations in testes
- Gene expression studies in testes
- Hormone measurements in testes and blood

In addition to these rat studies, an in vitro assay for analysis of PPAR α and PPAR γ transactivation was developed as described in appendix Y. Some of the chemicals tested in this assay were similar to those tested in vivo, as listed in Table 3.

Table 2. Purpose and main endpoints of in vivo studies.

The last column refers to papers presented in appendix X, and additional contributions presented in appendix Y.

Compounds (dose, mg/kg bw per day)	Animals	Purpose	Endpoints related to this thesis	Reference
DEHP (750), DEHP (750) + DEHA (400)	Fetal rats exposed in utero	Investigate combination effects and fetal testicular effects	Hormones, histology, apoptosis	I, II
DEHP (300), DEHP (750), DEHP (750) + DEHA (400)	Perinatally exposed prepubertal and adult rats	Investigate combination effects and antiandrogenic effects	Hormones, histology, apoptosis, immunohistochemistry	I, II, III
DEHP (300), DINP (750), DEHP (300) + DINP (750)	Fetal rats exposed in utero	Investigate combination effects and fetal testicular effects	Hormones, histology, immunohistochemistry	I + poster appendix Y
DEHP (300, 100, 30, 10)	Fetal rats exposed in utero	Investigate mechanism of action and dose- dependency	Hormones, histology, immunohistochemistry, gene expression	IV
DIBP (600)	Fetal rats exposed in utero	Investigate fetal testicular effects	Hormones, histology, immunohistochemistry gene expression	V + poster appendix Y
Prochloraz (150, 50)	Perinatally exposed fetal and prepubertal rats	Investigate mechanism of action	Immunohistochemistry	VI
Butylparaben (100), Perfluorooctanoate (20), Rosiglitazone (1)	Fetal rats exposed in utero	Test whether other PPAR ligands have similar effects to phthalates in vivo	Hormones, histology, gene expression	Appendix Y

Table 3. In vitro studies

Compounds	Type of study	Purpose	Reference
Butylparaben Propylparaben Perfluorooctanoate MEHP Rosiglitazone Prochloraz	PPAR reporter gene assay	Detect compounds interacting with PPAR α and PPAR γ	Appendix Y

My main contributions to paper III (DEHP and DEHA young and adult animals) and paper VI (Prochloraz) were the analyses of testicular histopathology and immunohistochemistry in fetal rats.

5 Results

The main results of the rat studies have been published in paper I to VI (Appendix X). Additionally, fetal testicular histopathology was examined in the combination study of DEHP and DINP, and gene expression was examined on DIBP-exposed fetal testes. The results of these studies can be found on the posters presented in Appendix Y as well as in the tables below. The results of the *in vivo* and *in vitro* studies on PPAR agonists (butylparaben, perfluorooctanoate, rosiglitazone) are described in appendix Y and summarized in Table 7.

Apoptosis assessment in the combination studies on DEHP and DEHA did not reveal any important effects (Paper II), and as this was the topic of my MSc thesis ⁵⁹, the results on apoptosis assessments will not be discussed thoroughly in this PhD thesis.

5.1 Comparison of *in vivo* studies

As study designs and endpoints were comparable for most of the studies, results of the different studies are summarized in Table 4 to Table 7. For simplicity, only the results in the highest dose group from dose-response studies are listed. DEHA did not appear to modulate the effects of DEHP and only results from the DEHP group in that study are listed here.

Testes of phthalate exposed rat fetuses exhibited some very characteristic alterations including the presence of multinuclear gonocytes, presence of enlarged tubules with centrally located gonocytes, clustering of Leydig cells with altered appearance and vacuolization of Sertoli cells, as listed in Table 4. DEHP-, DINP- and DIBP-exposed animals all exhibited these alterations to varying degrees.

Interestingly, the effects on gonocytes and Sertoli cells were not as prevalent on GD 19 as on GD 21 in testes of DIBP-exposed animals, while Leydig cell effects were present already at GD 19 (Table 4).

No histopathological effects were observed in fetal testes following exposure to prochloraz, butylparaben, PFOA, or rosiglitazone at GD 21.

Table 4. Histopathological alterations in fetal testes of phthalate-exposed rats.

Numbers indicate the percentage of exposed animals with a certain histological phenotype observed when examining one section per testis. The percentage of control animals with that phenotype is listed in parentheses. See papers II, IV and V for further explanations of the observed alterations.

Compound (dose, mg/kg bw/day)	Age of animal	Multinuclear gonocytes	Enlarged tubules with centrally located gonocytes	Clustering of Leydig cells with altered appearance	Sertoli cell vacuolization	Paper
DEHP (750)	GD 21	64% (0%)	100% (0%)	50% (12%)	100% (6%)	II
DEHP (300)	GD 21	100% (0%)	68% (0%)	72% (18%)		Poster, app. Y
DINP (750)	GD 21	83% (0%)	22% (0%)	52% (18%)		
DEHP (300) + DINP (750)	GD 21	95% (0%)	76% (0%)	70% (18%)		
DEHP (300)	GD 21	Yes	Yes	Yes	Yes	IV
DIBP (600)	GD 19	0% (8%)	22% (0%)	100% (15%)	11% (0%)	V
DIBP (600)	GD 21	62% (10%)	88% (0%)	87% (0%)	88% (0%)	

The three tested phthalates DEHP, DINP and DIBP all reduced fetal testosterone production and testicular testosterone content (Table 5). Concomitantly, serum testosterone levels were reduced, though not as significantly as testicular testosterone levels. As the masculinization of male external genitalia is stimulated by testosterone, compounds that impair testosterone production or -function can reduce the anogenital distance. This was observed with DEHP and DIBP, but was not assessed in the study on DINP. Serum luteinizing hormone (LH) was increased by DEHP in one study, while the observed tendencies to increases in LH levels were not statistically significant in the combination study on DEHP and DINP (Table 5).

Comparable reductions of testosterone levels and anogenital distance (AGD) in males were seen with prochloraz, but not with any of the other compounds tested.

In prepubertal and adult DEHP-exposed rats, tendencies to hormonal changes similar to those observed in DEHP exposed fetuses (i.e. reduced testosterone and increased LH levels) were observed, but the changes were not statistically significant.

Table 5. Changes in hormone levels and anogenital distance (AGD) in fetal male rats. Percent of control mean value (control mean = 100%).

T: testosterone, NS: not statistically significant. ↓ or ↑ indicates statistically significant reductions or increases of the listed parameters. # at PND 3

Compound (dose, mg/kg bw/day)	Age of animal	Plasma/serum T	Testicular T content	Testicular T production ex vivo	Plasma/serum LH	Change in AGD	Paper
DEHP (750)	GD 21	↓ 13%	↓ 23%	↓ 18%	↑ 216%	↓ 86% [#]	I
DEHP (300)	GD 21	70% NS	↓ 30%	↓ 21%	149% NS		I
DINP (750)	GD 21	71% NS	↓ 27%	↓ 31%	136% NS		
DEHP (300) + DINP (750)	GD 21	↓ 48%	↓ 16%	↓ 10%	↑ 186%		
DEHP (300)	GD 21	↓ 50%	↓ 21%	↓ 37%			IV
DIBP (600)	GD 19		↓ 30%	79% NS		↓ 85%	V
DIBP (600)	GD 20/21		↓ 9%	↓ 4%		↓ 84%	
Prochloraz (150)	GD 21	↓ 34%	↓ 17%	↓ 13%	158% NS	↓ 88%	VI
Butylparaben (100)	GD 21		105% NS	106% NS		↔	
PFOA (20)	GD 21		136% NS	115% NS		↔	
Rosiglitazone (1)	GD 21		109% NS	106% NS		↔	
DEHP (750)	PND 22	72% NS			571% NS		II
DEHP (750)	PND 190	68% NS	83% NS				

Immunohistochemistry reveals not only the localization of proteins in tissues, but also to some extent the amount of the examined protein. Some of the proteins involved in steroid synthesis were less abundant in testes with low testosterone production. Scoring of immunostaining intensity revealed that particularly P450scc, StAR and CYP17 were less abundant in testes from phthalate-exposed fetuses (Table 6). Additionally, all three phthalates decreased the immunostaining intensity for PPAR γ .

Prochloraz-exposed animals did not show any differences in immunostaining intensity for P450scc or StAR despite the observed reduction of testosterone synthesis. On contrary, the enzyme CYP17 appeared to be upregulated in prochloraz-exposed testes. The intensity of 17 β HSD (17 β -hydroxysteroid dehydrogenase) type 10 appeared to be reduced in testes of prochloraz-exposed rats (Table 6).

Table 6. Summary of effects on immunohistochemistry in fetal rat testes (GD 21).

↓, ↑ or ↔ indicates reduced, increased or unchanged intensity of immunostaining in Leydig cells of exposed rat fetuses compared to controls. * indicates results published in the listed papers, while the remaining results are yet unpublished.

Compound (dose, mg/kg bw/day)	PPAR γ	Star	PBR	P450scc	3 β HSD	CYP17	17 β HSD type 10	Paper
DEHP (750)					↔*			I
DEHP (300)	↓	↓		↓	↔	↓		
DINP (750)	↓	↓		↓	↔	↓		
DEHP (300) + DINP (750)	↓	↓		↓	↔	↓		
DEHP (300)	↓*	↓*	↓*	↓*		↓		IV
DIBP (600) GD 19	↓					↓		V
DIBP (600) GD 21	↓	↓*		↓*		↓		
Prochloraz (150)	↔	↔*		↔*	↔*	↑*	↓*	VI
Butylparaben (100)	↔	↔	↔	↔				
PFOA (20)	↔	↔	↔	↔				
Rosiglitazone (1)	↔	↔	↔	↔				

Corresponding to the results on immunohistochemistry for factors involved in steroid synthesis, mRNA levels of genes coding for these factors (SR-B1, StAR, PBR, P450scc, CYP17) were reduced in testes of phthalate-exposed fetal rats (Table 7). In contrast to the immunostaining results, PPAR γ mRNA levels were unaffected by DEHP or DIBP, whereas PPAR α mRNA levels were reduced at GD 19 in testes of DIBP exposed animals. No statistically significant effects of prochloraz on mRNA levels were observed for any of these factors (Table 7). The discrepancies between results of immunostaining and gene expression studies may indicate that the observations of increased CYP17 protein levels in prochloraz-exposed animals and decreased PPAR γ protein levels in phthalate-exposed animals are due to effects at protein level rather than at transcription level. Alternatively, changes in gene expression may occur at an earlier age than the changes in protein expression are observed.

In addition to the listed effects, testicular mRNA levels of SF1 and InsI3 were reduced by DEHP and DIBP, but were not measured in studies on other test compounds.

Table 7. Summary of effects on gene expression in fetal rat testes (GD 21).

↓, ↑ or ↔ indicates reduced, increased or unchanged mRNA levels in testes of exposed rat fetuses compared to controls. * indicates results published in the listed papers, while the remaining results are yet unpublished

Compound (dose, mg/kg bw/day)	PPAR γ	PPAR α	SR-B1	Star	PBR	P450scc	CYP17	Paper
DEHP (300)	↔*	↔*	↓*	↓*	↓*	↓*	↔*	IV
DIBP (600) GD 19	↔	↓	↓*	↓*	↔*	↓*	↓*	Poster, app. Y
DIBP (600) GD 21	↔	↔	↓*	↓*	↔*	↓*	↓*	
Prochloraz (150)			↔*	↔*		↔*	↔*	VI
Butylparaben (100)	↔	↔	↔	↔		↔	↔	
PFOA (20)	↔	↔	↔	↔		↔	↔	
Rosiglitazone (1)	↔	↔	↔	↔		↔	↔	

5.2 Comparison of PPAR transactivation in vitro with effects on fetal testosterone levels in vivo

Reporter gene assays were developed in order to detect chemicals transactivating PPAR α and/or PPAR γ . As mentioned, certain PPAR α - or PPAR γ -agonists can also reduce testosterone production in rats. In Table 8, the ability of chemicals to transactivate PPAR α or PPAR γ in vitro is compared to their ability to reduce fetal testicular testosterone levels in vivo. DEHP reduced testosterone production in vivo, and its primary metabolite MEHP was also able to transactivate PPAR α and PPAR γ in vitro. Prochloraz did not appear to activate PPARs, but was able to reduce fetal testicular testosterone levels in vivo. Butylparaben, PFOA and rosiglitazone transactivated PPAR α and PPAR γ to varying degrees, but did not affect testosterone production in fetal male rats at the applied doses, although similar doses had been found to reduce testosterone levels in young or adult rats in other studies^{45;54}.

This comparison does not indicate any association between PPAR activation and fetal testosterone production, but cannot rule out the possibility of a connection either.

Table 8. Comparison of results of in vitro studies on PPAR α and PPAR γ transactivation and in vivo studies on fetal testicular testosterone production (see appendix Y for further description).

+++ indicates a high degree of effect, + indicates a moderate degree of effect, and - indicates no effect of the compound on the listed endpoint at the applied dose- or concentration levels.

Compound (dose in vivo, mg/kg bw/day)	PPAR α transactivation	PPAR γ transactivation	Reduces fetal testicular testosterone levels in vivo	Paper (in vivo data)
MEHP/DEHP (300)	+++	+++	+++	IV
Prochloraz (150)	-	-	+++	VI
Butylparaben (100)	+	+	-	Appendix Y
PFOA (20)	+++	+	-	Appendix Y
Rosiglitazone (1)	+	+++	-	Appendix Y

6 Discussion

6.1 *DINP and DIBP have similar effects as DEHP in fetal rat testis*

In order to examine whether DINP and DIBP are safer substitutes for the reproductive toxicant DEHP, we compared the effects of these compounds on fetal testicular testosterone production and testicular development. Prenatal exposure to DEHP, DINP and DIBP reduced testosterone levels and induced fetal testicular histopathological effects similar to those observed with DEHP (Table 4 and 5) and described for DBP by other groups^{33,34}. Multinucleated germ cells, enlarged chords with centrally located germ cells, large clusters of small Leydig cells, and Sertoli cells with cytoplasmic vacuolization were present in most DEHP-, DIBP- or DINP-exposed animals (Paper II, V and appendix Y). As these effects in fetal life are related to adverse long-term reproductive effects (discussed in section 6.2), this indicates a reason for concern for the use of DINP and DIBP as substitutes for DEHP and DBP.

The observations in young and adult DEHP-exposed rats (Paper III) confirm the findings described for DBP by other groups. As for DBP^{60,61}, severe degeneration of seminiferous tubules was seen in some animals, while others appeared largely normal. Some of the effects observed with DEHP and DBP have not been described for any other types of chemicals, namely the presence of dysgenetic tubules with intratubular Leydig cells in areas of Leydig cell hyperplasia. It is therefore remarkable that this phenomenon is not solely a result of DBP exposure, but also occurs with DEHP (Paper II).

Our studies on DIBP and DINP investigated endpoints in male fetuses and the effects may not be considered adverse as such. However, these effects on fetal histopathology and testosterone production are likely related to the adverse effects observed in adult phthalate-exposed rats as discussed in the following.

6.2 *Temporal differences in the toxic effects of phthalates*

Fundamentally, the most important effects caused by chemical exposure of experimental animals are adverse effects with relevance to humans. For the phthalates, the adverse and relevant effects seen in rats are hypospadias and low sperm quality. Reduced anogenital distance and presence of nipples in males are also characteristic signs of antiandrogenicity, but these endpoints are less well related to adverse effects in humans. Cryptorchidism and degeneration of epididymis and seminiferous tubules are likely causal factors for the low sperm quality. But what causes these malformations of reproductive organs? And why are some animals severely affected while others appear completely normal?

The testosterone peak in late gestation is essential for the normal formation of the external genitalia, and Insl3, a hormone produced in Leydig cells, appears to be essential for testicular descent at birth, and some of the effects of phthalates can be explained by reduced production of these two hormones³². In the following will be discussed how the testicular and epididymal degeneration may be related to the observed fetal effects and what may be causing the low testosterone and Inls-3 levels.

6.2.1 How is fetal testicular histopathology related to impaired spermatogenesis later in life?

Leydig cells of phthalate-exposed fetal rats are located in large clusters centrally in the testes and have smaller cytoplasm and smaller, more irregular nuclei than normal testes. This “inactive” appearance of the Leydig cells is very likely related to the low testosterone levels in phthalate-exposed males at this age. The peak in testosterone levels in late gestation is considered to be essential for the normal masculinization of males, as androgen receptors in the reproductive organs induce growth and differentiation of tissues⁶². Thus, the link between fetal Leydig cell effects and the effects on reproductive organ weights (prostate, levator ani/bulbocavernosus muscle) later in life is quite clear (Paper III).

Also the changes in the morphology of the testicular interstitial space and the occurrence of Sertoli cells outside the seminiferous tubules in fetal testes may be related to the development of dysgenetic tubules later in life, as discussed in Paper II. The alteration of interstitial “architecture” in fetal testes has also been observed with DBP and was related to the formation of incomplete tubules formed within dysgenetic areas at PND 4⁶¹. In that study it was shown that Leydig cells could be “trapped” inside the tubular structures and affect the function of Sertoli cells and spermatogenesis in that section of the seminiferous tubule later in life⁶¹. In our studies as well as the DBP studies, apparently normal testicular tissue surrounds the dysgenic areas in prepuberty and adulthood. These testes have likely gained full function and are capable of producing normal sperm. However, certain parallels can be drawn between the dysgenetic areas in phthalate-exposed rat testes and the development of testis cancer in humans. In human testes, Sertoli cells in areas with cancer precursor carcinoma-in-situ cells are immature and do not express certain markers of Sertoli cell maturity. Likewise, these markers are absent in dysgenetic areas of testes of DBP-exposed rats^{61;63}. If human testis cancer development is indeed related to Sertoli cell dysfunction, it may be speculated that the disruption of Sertoli cell function in dysgenetic areas of phthalate-exposed rat testes is a sign of a relationship between phthalate exposure and human testis cancer development.

In contrast to the effects on Leydig and Sertoli cells, the observed alterations of gonocytes in fetal testes are not as readily related to the effects observed later in life. Multinucleated germ cells are not observed later than PND 7 with a maximal incidence at GD 21^{60;64}. Despite the apparent increase in germ cell numbers and enlargement of seminiferous chords at GD 21, our studies did not indicate any alterations in the expression of apoptosis and proliferation markers at GD 21 in DEHP exposed testes (Paper II).

In a study on prenatal exposure to DBP, reduced germ cell numbers were observed from GD 21 to PND 25⁶⁵. Increased expression of markers for proliferation and apoptosis was observed at GD 17, but not GD 21⁶⁵. Thus, the low germ cell number at GD 21 observed in that study was explained by an increased apoptosis rate around GD 17. The low germ cell numbers observed postnatally were explained by a low proliferation rate, which was seen at PND 6⁶⁵. At PND 25, the proliferation rate increased and germ cell numbers were restored in adulthood in scrotal testes. Collectively, the changes in germ cell numbers in scrotal testes appeared to be transient in rats and thus not directly related to the testicular toxicity of these phthalates. This conclusion is also supported by our studies showing no considerable changes of apoptosis rates at GD 21, PND 22, 27 or in adulthood in most testes of DEHP-exposed rats (Paper II).

In our studies, prepubertal testes (PND 22) of DEHP exposed rats were slightly smaller and appeared slightly delayed in their development, similarly to the findings with DBP described by Ferrara (2005)⁶⁵ and Barlow & Foster (2003)⁶⁰. This may be related to delayed Sertoli cell development, as also serum levels of the Sertoli cell product Inhibin B was reduced by DEHP at PND 22 (Paper I). These Sertoli cell effects appeared to be transient, as there was no general reduction of testis size, and testis size reflects the number and function of Sertoli cells⁶³. Furthermore, in adult DEHP-exposed testes sperm quality and histopathology appeared largely normal in most adult testes in our study (Paper III).

A few DEHP-exposed testes, however, were completely degenerated. Likewise, a clear difference between scrotal and cryptorchid testes of exposed animals was found in the DBP study⁶⁵. This difference emerged postnatally, as germ cell numbers of scrotal and cryptorchid testes of DBP exposed animals were similar until PND 25, whereas cryptorchid testes did not exhibit full spermatogenesis in adulthood⁶⁵.

However, other studies have shown that degeneration in adult testes was not associated with cryptorchidism, but rather with epididymal malformation. Barlow & Foster (2003) examined the pathogenesis of testicular and epididymal effects of prenatal DBP exposure of rats from fetal life to adulthood. Fetal epididymides of DBP-exposed animals exhibited decreased coiling of ducts - an effect, which progressed to malformation and degeneration in adulthood⁶⁰. As certain other antiandrogenic compounds such as linuron have similar epididymal effects as listed in Table 1, this may be related to low testosterone production or -function. In contrast to the epididymal effects, the testicular effects of DBP observed in late gestation (multinucleated gonocytes, increased numbers of gonocytes and clustering of Leydig cells) resolved, while a different set of morphological alterations appeared later on (focal dysgenesis in the seminiferous epithelium in some testes and general degeneration in others)⁶⁰, as also seen in our DEHP study. The severity of testicular degeneration progressed with age and was suggested to result from the progressing epididymal lesions causing obstruction of flow from the testes⁶⁰.

In our study on DEHP, the observed testicular degeneration in a few testes was to some degree associated with epididymal degeneration, as four of the six animals with degenerated testes also had small epididymides (Paper III). It is possible that the two remaining animals with small testes were in fact cryptorchid, thus explaining the testicular degeneration, but we did not register whether testes were cryptorchid or not in that study. Apparently, there are rat strain differences in the response to DBP, as Sprague-Dawley rats mainly develop epididymal effects^{27;60} whereas Wistar rats rather become cryptorchid⁶¹.

Apparently, two or three pathways may be leading to the testicular degeneration later in life, as illustrated in Fig. 4. Low *Ins13* and/or testosterone production may induce cryptorchidism, which prevents normal testicular function and is a risk factor for testis cancer and low sperm count. Low testosterone production by Leydig cells may either induce epididymal malformation causing testicular degeneration or impair Sertoli cell function. Impaired Sertoli cell function or a reduced number of Sertoli cells is known to reduce the number of germ cells and thus sperm production. The characteristic “all or none” response with regard to testis degeneration after perinatal phthalate exposure may indicate that the subtle effects on Sertoli cell function are transient and probably not causally related to the testicular degeneration.

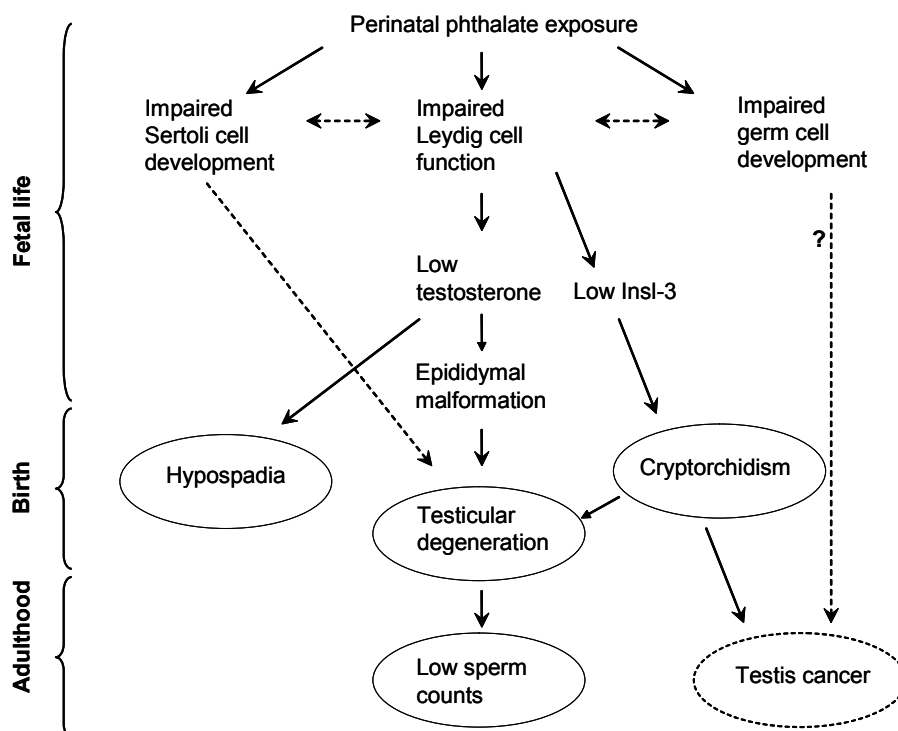


Fig. 4. Development of the adverse effects of phthalates related to the testicular dysgenesis syndrome. Perinatal phthalate exposure induces hypospadias, testicular degeneration, low sperm counts and cryptorchidism in rats, while testis cancer is not seen in rats but is causally related to cryptorchidism in humans. Arrows indicate well-described relationships between these effects, while dotted arrows indicate more uncertain relationships.

Collectively, the impaired fetal testosterone and InsI3 production are central events in the development of adverse effects of perinatal phthalate exposure. It is also possible that the alterations of germ cell development and fetal testicular structure may influence adult reproductive function. Thus, our observations of reduced testosterone production in addition to alterations of fetal testicular histopathology in testes of DINP- and DIBP-exposed rats indicate that adverse effects of perinatal exposure can be expected in adulthood.

6.2.2 Prepubertal and adult phthalate exposure

This thesis deals mainly with the effects of perinatal phthalate exposure, but phthalates are also toxic to the testes of young and adult rats. As discussed in Paper II, phthalate exposure disrupts Sertoli cell structure and function and leads to apoptosis of germ cells. The direct interactions leading to these effects are not clear, but among the observed events are interferences with Sertoli cell proliferation and induction of germ cell apoptosis by disruption of signals from Sertoli cells, as well as disruption of gap junctional intercellular communication between Sertoli cells³². Due to the persistence of stem cell spermatogonia, the effects on spermatogenesis seen with prepubertal or adult phthalate exposure appear to be reversible when Sertoli cells recover after ended exposure⁶⁶.

Collectively, the prenatal or early postnatal period appears to be more sensitive to phthalate exposure than prepuberty or adulthood in rats. Children may also be exposed to higher levels of phthalates than adults, as

will be discussed later. This, together with the increased sensitivity, emphasizes why the major concern for adverse effects is with perinatal phthalate exposure.

6.3 Phthalate mechanisms of action in fetal rat testes

As previously mentioned, perinatal exposure to DBP, DEHP, BBP or DINP was found to induce adverse reproductive effects in rats¹⁷. Recently, Liu et al (2005) has compared the gene targets of different phthalates and found that DBP, DEHP, BBP and dipentyl phthalate (DPP) all reduced anogenital distance and affected the expression of a common group of genes in fetal testes, while DEP, DMP and DOTP did not affect anogenital distance and altered the testicular expression of a different group of genes⁶⁷. Mechanistic studies on phthalate mechanism of action have mainly been investigated with perinatal DBP exposure as described in numerous papers, and due to the mentioned similarities for the antiandrogenic phthalates, these mechanisms can be regarded as representative for other antiandrogenic phthalates as well.

6.3.1 Protein and gene expression changes causing low fetal testicular testosterone levels

In addition to measuring testicular testosterone content and –production, the testicular expression of proteins and genes involved in steroid synthesis was assessed in fetal rats exposed to phthalates (Paper IV and V). In correspondence with the low testosterone production, mRNA expression levels of genes coding for factors related to steroid synthesis were reduced in fetal testes of DEHP- and DIBP-exposed animals (Table 7, Fig. 5). Immunohistochemistry can be used as a marker for protein levels of these factors, although quantification can be difficult. Our results showed reduced protein levels of StAR and P450scc in testes of fetal rats exposed to DEHP, DIBP, or DINP (Table 6). In DEHP-exposed testes, reduced protein and mRNA levels were observed for PBR, a protein that assists StAR in the mitochondrial cholesterol transport. The downregulation of PBR expression by DEHP in fetal rat testes had not previously been described, but PBR was reduced in testes of DEHP exposed adult mice in a study by Gazouli et al., 2002⁴¹. Lehmann et al. (2004) found that DBP reduced PBR protein expression and increased *PBR* mRNA levels in fetal rat testes⁶⁸. *StAR*, *P450scc*, and *SR-BI* appeared to be more sensitive to phthalate exposure than the other genes examined, as mRNA expression for these three genes were reduced by 300 mg/kg of DEHP as well as by 600 mg/kg of DIBP. In contrast, *P450c17* was only affected by DIBP, and *PBR* was only affected by DEHP. At 100 mg/kg bw/day of DEHP, only the reduction of *StAR* expression was statistically significant, indicating that *StAR* is more sensitive than the other genes. A similar pattern was described by Lehmann et al (2004)⁶⁸, who found statistically significant effects of DBP on *StAR*, *P450scc*, and *SR-BI*, but not *P450c17* at doses down to 50 mg/kg per day. However, the relative sensitivities of these genes appear to vary between different studies on DBP^{35;42;67-69}.

In our study on DIBP, the effects on gene expression were generally more marked at GD 19 than at GD 21, whereas testosterone levels were clearly more reduced at GD 21 than GD 19 (Paper V). Similarly, a study on DBP showed that *P450scc* and *P450c17* expression levels were only reduced at GD 19 and not at GD 21³⁵. On the contrary, *StAR* and *SR-BI* expression levels were reduced more at GD 21 than GD19 in that study³⁵. At GD 16, only *StAR* and *SR-BI* expression levels were reduced, when *P450scc* and *P450c17* expression

levels were similar to controls³⁵. Overall, the downregulation of genes coding for factors involved in steroid synthesis may precede the low testosterone levels, but in order to search for factors inducing the low steroid synthesis, it may be necessary to examine gene expression even before GD 19.

In a study by Thompson et al. (2004), the exposure period was narrowed down to one single day and effects on testosterone synthesis related genes were examined⁴². It was shown that effects of treatment with DBP on GD 19 only were comparable to the effects of exposure from GD 12 to GD 19⁴². Treatment from GD 12 to GD 18 induced similar effects as GD 12 to GD 19 treatment, while the effects observed at GD 17 were subtle⁴². This indicates that GD 17 to 19 is a critical time period for repressing testosterone and gene expression levels with DBP. Another study by Thompson et al (2005) revealed that exposure to one dose of DBP on GD 19 reduced testosterone levels already 1 hour after exposure by oral gavage, while reduced expression of *SR-B1*, *StAR*, *P450scc*, and *P450c17* was not evident until 6 hours after exposure⁶⁹. Interestingly, *StAR* expression was downregulated already after 3 hours further indicating a central role for this protein in the series of events. From 12 hours onwards, testosterone levels were reduced even further⁶⁹. Apparently, there is an initial direct effect of DBP on testosterone production and later an indirect effect caused by reduced expression of genes and proteins involved in steroidogenesis. The authors speculate that the acute effect may be due to interaction with a fetal Leydig cell specific transcriptional regulator⁶⁹.

Collectively, the effect of phthalate exposure on StAR expression and cholesterol transfer across the mitochondrial membrane to P450scc may be central in the chain of events leading to reduced testosterone production (Fig. 5). As reviewed by Stocco et al., 2005, the regulation of StAR expression involves a very complex pathway and can be divided into an acute and a chronic response⁷⁰. Hypothetically, many factors can thus be suggested as targets for phthalates. The acute regulation of StAR function is via LH/hCG stimulation activating the cAMP/protein kinase A pathway by which phosphorylation of the StAR protein induces changes in steroid synthesis within minutes⁷⁰. Additionally, cAMP is involved in regulating StAR expression levels, but several cAMP-independent regulators of StAR expression have also been described⁷⁰. The more chronic regulation of *StAR* gene expression (i.e. changes within hours) involves activation of specific membrane receptors by growth factors inducing different intracellular pathways in addition to the nuclear receptor steroidogenic factor-1 (SF1), which acts as a transcription factor for the *StAR* gene^{70;71}. An effect of phthalates on these receptors could in part explain the downregulation of StAR in fetal testes. According to Liu et al. (2006), the complex formation of StAR and PBR is central in the acute regulation of steroidogenesis, and disruption of this complex reduces hCG-induced steroid synthesis dramatically⁷². It may be hypothesized that phthalates interact with StAR, PBR and/or other proteins in the complex, thereby preventing its interaction with and transport of cholesterol.

Reduced fetal testicular *SF1* expression following phthalate exposure (Paper IV) has not previously been described. In Fig. 5 an interaction between phthalate metabolites and SF1 is suggested, but in a study by Thompson et al., 2004, no interaction between SF1 and the DBP metabolite monobutyl phthalate (MBP) was observed⁴². This does not exclude the possibility that other phthalate metabolites may be able to interact with SF1.

In summary, several hypotheses can explain the testosterone-reducing effects of phthalates in late gestation, but it remains speculative which mechanism is true. Fig. 5 illustrates the possible mechanisms by which nuclear receptors may be involved in the downregulation of testosterone production by phthalates. Additionally, the two-step downregulation of testosterone levels in fetal DBP-exposed testes described by Thompson et al. (2004) points to different mechanisms causing the acute versus the long-term downregulation of steroidogenesis⁴².

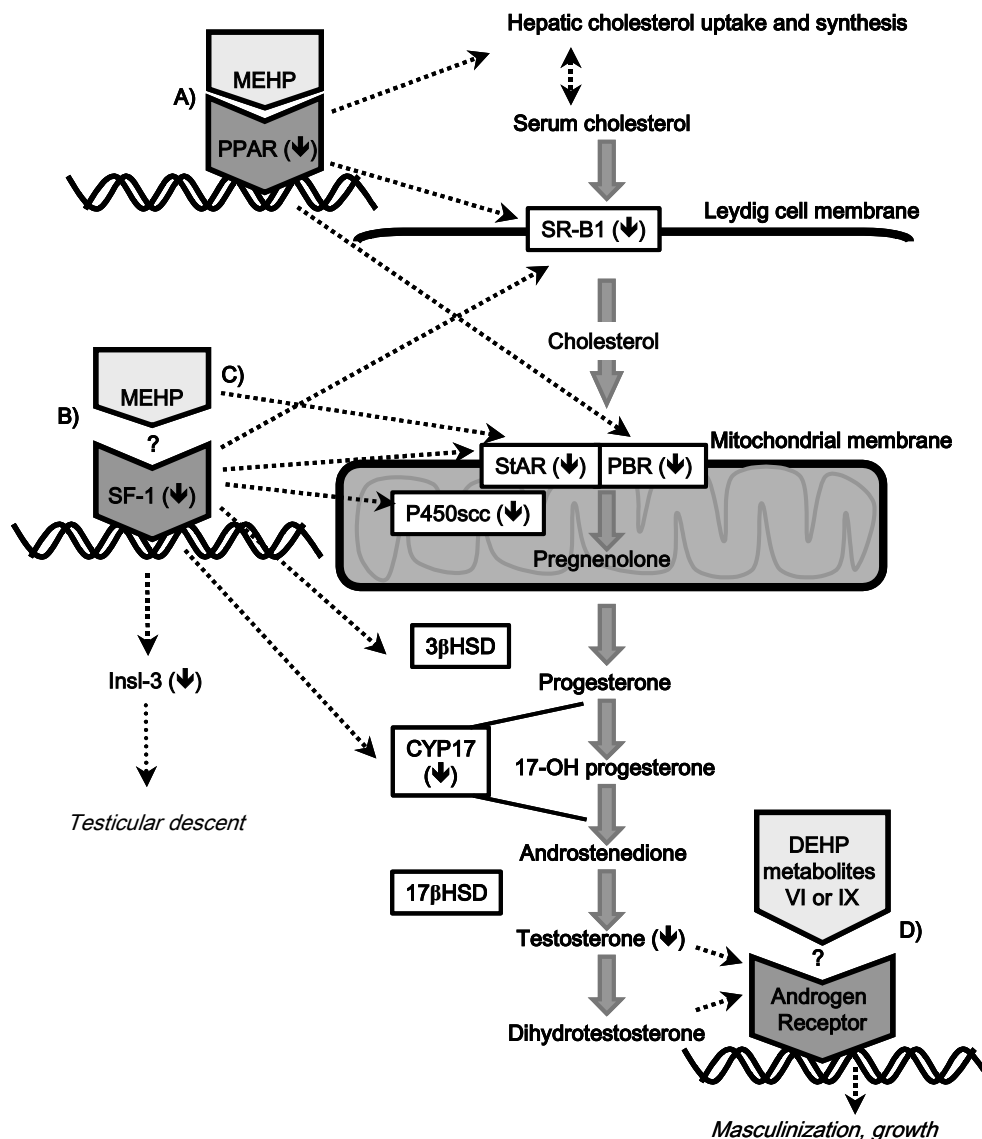


Fig. 5. Model of phthalate mechanisms of action.

A) MEHP is known to interact with PPARs, which are involved in regulation of cellular and mitochondrial cholesterol uptake. This interaction may be related to the reduction of testosterone production by phthalates.

B) It may be hypothesized that MEHP or other metabolites interact with SF1, a regulator of several steps in steroidogenesis, thereby reducing testosterone production.

C) It may be hypothesized that MEHP or other metabolites interrupts the function of StAR or the related mitochondrial cholesterol transport complex, as this StAR is an acute regulator of steroidogenesis.

D) Studies have shown that the DEHP metabolites VI and IX interact with the androgen receptor in vitro³¹.

(↓) indicates factors reduced (at protein or gene expression level) in fetal rat testes by DEHP or DBP.

Dotted arrows indicate pathways of regulation of various steps in steroid synthesis and androgen function.

In addition to the described indirect effects on mitochondrial cholesterol uptake and steroid synthesis, phthalates may directly inhibit enzymes in steroidogenesis, affect the metabolism of testosterone, or alter the systemic cholesterol availability or lipid metabolism. Fan *et al.* (2004) showed an upregulation of steroid metabolic P450 enzymes in rat livers after exposure to DBP or other PPAR agonists, indicating increased steroid breakdown⁷³. However, increased hepatic steroid breakdown is not likely the primary mechanism of reduced testosterone levels by phthalates, as culturing primary Leydig cells with phthalates reduces testosterone production directly^{41;74}. Similarly, the current findings of reduced testicular testosterone content and testosterone production *ex vivo* following phthalate exposure demonstrate that the possible influence of increased hepatic steroid breakdown is limited.

6.3.2 *Ins13* regulation by nuclear receptors

As previously mentioned, *Ins13* production is critical for the normal male development of mice and rats, in particular testicular descent. In humans, mutations of the *Ins13* gene are related to symptoms of the testicular dysgenesis syndrome, but the exact role of *Ins13* has not been determined^{75;76}. *Ins13* is produced by the Leydig cells, and similarly to the factors involved in steroidogenesis the transcription of *Ins13* is regulated by SF1⁷⁷. Additionally, the orphan nuclear receptor Nur77 regulates *Ins13* transcription⁷⁸, but it is not known whether SF1 or Nur77 are involved in the pathways by which phthalates affect *Ins13* levels (Fig. 5). Maternal exposure to the synthetic estrogen diethylstilbestrol induces cryptorchidism in the offspring of humans as well as rodents. In *mice* prenatally exposed to diethylstilbestrol, testicular *Ins13* mRNA expression was reduced at GD16 and GD18, whereas *SF1* expression was unaltered, indicating an SF1-independent regulation of *Ins13*⁷⁹. In contrast, *SF1* expression was reduced on GD18 in *rats* prenatally exposed to diethylstilbestrol⁸⁰. Thus, it remains to be elucidated by which mechanism the expression of *Ins13* is downregulated by chemical exposure.

6.3.3 Involvement of PPARs?

PPARs are known to regulate factors involved in cellular and mitochondrial cholesterol transport as well as hepatic cholesterol metabolism, and as phthalates are known to activate PPARs a role for these receptors in the downregulation of fetal steroidogenesis can be hypothesized (Fig. 5).

This hypothesis was based on the fact that the degree of testis toxicity of various phthalates correlates well with their potency of PPAR activation⁴⁴. Additionally, other PPAR α or PPAR γ ligands affect steroid production in testes and ovaries of rats and humans *in vitro* and *in vivo*^{41;45-49}. Several PPAR agonists (phthalates, ammonium perfluorooctanoate, clofibrate, WY14,643) have been found to reduce testosterone production in primary Leydig cell culture^{41;45;48}. Neonatal exposure of rats to clofibrate reduced testosterone levels in adulthood⁴⁷, and ammonium perfluorooctanoate exposure reduced hCG-stimulated testosterone production in adult male rats⁴⁶. The PPAR γ agonist rosiglitazone is known to reduce testosterone levels in normal men⁴⁹. Rosiglitazone is used for treatment of women with polycystic ovary syndrome and reduces hyperandrogenism in these patients^{52;53}. Thus, it is plausible that the testicular effects in rats exposed to PPAR-activating environmental compounds are also of human relevance.

The impact of phthalate exposure on PPAR expression was studied as described in Paper IV. Changes in fetal testicular *PPARα* and *PPARγ* mRNA levels and *PPARγ* protein expression was assessed at GD 21 in DEHP-exposed rats. Animals exposed to 300 mg/kg bw per day of DEHP had a reduced intensity of immunostaining for *PPARγ* in fetal Leydig cells, indicating reduced *PPARγ* protein levels compared to controls (Paper IV). Similarly, *PPARγ* staining was reduced in Leydig cells at GD 19 and 21 in rats exposed to DIBP and at GD 21 in DINP exposed rats (Table 6). The reduced Leydig cell staining for *PPARγ* in phthalate-exposed fetal testes may point to an involvement of *PPARγ* in the mechanism of action of phthalates. However, no alterations in mRNA levels of *PPARγ* were observed in DEHP- or DIBP-exposed testes at GD 21 (Table 7 and Paper IV). DIBP-exposed fetal testes had lower levels of *PPARα* on GD 19, but not GD 21. If PPARs are indeed involved in the mechanisms of action of phthalates, it may be relevant to examine changes in gene expression even before GD 19.

No previous studies have described the distribution of PPARs in fetal testes. Furthermore, the distribution of PPARs in *adult* testes has been poorly described with one study describing weak *PPARγ* expression (based on in situ hybridization) in rat testes⁸¹ and another study describing absence of *PPARγ* mRNA in mouse Leydig tumor cells⁴¹. *PPARα* expression, however, has been detected in human and rat testes as well as mouse Leydig tumor cells^{41;81;82}. Our studies have shown *PPARγ* staining also in Leydig cells of prepubertal and adult rat testes. As this staining pattern partly contradicted the observations described in the literature, we confirmed these findings using a monoclonal *PPARγ* antibody from another manufacturer showing similar staining of rat Leydig cells at various ages (results not shown). These studies appear to strengthen the evidence that *PPARγ* is present in fetal Leydig cells. However, the results regarding the impact of phthalate exposure on testicular PPAR protein and gene expression show that protein expression changes are not always preceded by detectable gene expression changes. It is possible that PPAR interactions may be involved in the downregulation of testosterone production in phthalate-exposed rats without any measurable changes in *PPAR* gene expression.

In addition to the nuclear receptors SF1 and the PPARs, cells contain several orphan nuclear receptors, some of which may be involved in the regulation of steroidogenesis. One example is the nuclear receptor DAX-1, which interacts with SF, AR and the estrogen receptor and regulates the transcription of several genes for factors involved in steroid synthesis including *Star*, *P450scc*, *3βHSD* and *CYP17*⁸³. The testicular expression of *DAX-1* mRNA is reduced by phthalate exposure⁶⁷, but it is presently unknown whether DAX-1 or other orphan nuclear receptors interact with phthalates and whether they are related to the testosterone- and InsI3-reducing effect of phthalates.

6.3.4 In vitro studies on phthalate mechanism of action

In vitro studies have certain advantages compared to animal studies, as they can be more informative about mechanisms of action. Steroidogenesis can be studied in various Leydig cell lines, though the Leydig tumor cell lines do not produce high levels of testosterone. Instead, progesterone levels can be assessed. Gazouli et al. (2002) observed reduced progesterone production in MA-10 mouse Leydig tumor cells and R2C rat Leydig tumor cells incubated with 10 μM of MEHP⁴¹. Recent studies in mouse Leydig tumor cells (MLTC-

1) has shown that hCG-stimulated progesterone production was decreased with high levels (50 to 800 μM) of MBP, the monoester metabolite of DBP, while low levels of MBP (1 nM to 1 μM) increased progesterone production⁸⁴. Additionally, these studies showed reduced StAR expression at high MBP concentrations and increased StAR protein and mRNA expression at lower concentrations corresponding to the effects on progesterone levels and further demonstrating an important role of StAR in the mechanism of action of phthalates⁸⁴.

In isolated adult rat Leydig cells 1 μM of MEHP reduced testosterone production⁴¹, whereas studies in primary cultures of fetal rat Leydig cells have not been able to reveal any effects of phthalates or their monoesters on testosterone production⁸⁵. A study on DEHP and some of its metabolites (MEHP, metabolite VI and XI) on fetal rat testis in culture showed no effects on testosterone production with doses up to 1 or 10 μM ⁸⁵. In that study, Stroheker et al. (2006) measured concentrations of DEHP metabolites in testes of DEHP-exposed rats and aimed to apply similar concentrations to a fetal rat testis culture. An average concentration of 37 nmol/g tissue of DEHP-metabolites were measured in DEHP-exposed fetal testes, and this was considered to correspond to a “local” concentration of 37 μM present at the cellular level⁸⁵. However, they only tested up to 1 μM of MEHP in vitro, which is much lower than the proposed “local” concentration. Thus, the concentrations applied in the study on fetal rat testis culture may be too low to observe an effect of the DEHP metabolites.

The study by Stroheker et al. (2006) interestingly enters a field of phthalate-research that has not yet been investigated thoroughly. New studies on the metabolism of DEHP reveal that the well-investigated primary metabolite MEHP may not be the most prevalent or active metabolite in vivo. Rather, secondary oxidized metabolites have longer half-lives and may even be able to accumulate in the body¹⁴. Some of the in vitro studies previously made with MEHP may need to be repeated with these secondary metabolites and may reveal effects that cannot be found with MEHP. For example, it was previously mentioned that none of the reproductive toxic phthalate diesters or their primary monoester metabolites are strong AR antagonists in vitro. However, two metabolites of DEHP, metabolite VI and IX have been shown to antagonize the AR weakly³¹.

The studies in Leydig tumor cells point to effects of phthalates directly on the Leydig cells, and in particular on mitochondrial cholesterol uptake by StAR and/or PBR. In the above mentioned study by Gazouli et al. (2002), *PBR* mRNA levels were reduced by DEHP in MA-10 cells. It was concluded that the reduced progesterone level in MA-10 cells could not be due to *PPAR* γ interactions, as no *PPAR* γ mRNA could be measured in this cell type⁴¹. They concluded that *PPAR* α mediated the downregulating effect of MEHP on the cholesterol transporter PBR, as DEHP reduced PBR expression in wild-type mice, but not in *PPAR* α knockout mice⁴¹. As cholesterol transport by PBR is essential for steroid synthesis, the authors thus suggested that *PPAR* α may be responsible for the effect of MEHP on testosterone production. On the other hand, the lack of *PPAR* γ in MA-10 cells does not exclude the possibility that this cell type differs from rat Leydig cells, which (according to our studies) do express *PPAR* γ . The study did not reveal whether *PPAR* γ was expressed in the applied rat R2C Leydig tumor cells⁴¹.

In order to study whether the effect of phthalates is related to PPARs, it would be relevant to investigate if concomitant incubation of Leydig cells with a PPAR antagonist would decrease the effect of the phthalate. Additionally, if other PPAR agonists or antagonists affect the same steroidogenic targets as the phthalates in vitro, this may further strengthen the hypothesis of involvement of PPARs in the phthalate mechanism of action. Studies on the effects of PPAR α ligands versus PPAR γ ligands on steroid production in Leydig tumor cell lines and fetal rat Leydig cell culture may further elaborate on which PPAR subtype may be involved.

If PPAR-activating chemicals do indeed affect fetal testosterone production, this may be of concern for human reproduction, as humans are exposed to several types of chemicals interacting with PPARs⁵⁰. Additionally, it may be hypothesized that PPAR activation by endogenous sources (e.g. imbalances in the metabolic system) may be related to symptoms of the testicular dysgenesis syndrome, as has been suggested for phthalates. In line with this, research in the possible links between metabolic disorders and reproductive function has recently been attracting increased attention⁸⁶.

6.4 Other chemicals affecting testosterone production in vivo

6.4.1 In vitro and in vivo studies on PPAR activating chemicals

The hypothesis of involvement of PPARs in the mechanism of phthalate toxicity inspired our study on fetal testicular testosterone production after exposure to the PPAR agonists PFOA and rosiglitazone, which are known to reduce testosterone production in young or adult rats or humans. Additionally, we wished to examine whether other chemicals with effects on testosterone synthesis (i.e. parabens) were able to interact with PPARs.

Reporter gene assays for PPAR interaction revealed that butylparaben and propylparaben were weak activators of PPAR α and PPAR γ in vitro (Appendix Y). PFOA transactivated PPAR α rather strongly and was a weak activator of PPAR γ , while MEHP was a strong activator of both PPAR α and PPAR γ , supporting the findings by other groups^{43,50}. Prochloraz did not appear to transactivate either PPAR subtype. Antagonist assays were performed by exposing cells to test compounds concomitantly with the specific control ligands WY14,643 or rosiglitazone. Butylparaben appeared to be an antagonist of PPAR γ , and prochloraz appeared to antagonize both PPAR α and PPAR γ . Little is known about the endogenous ligands for PPARs or the human tissue levels of parabens, and it is thus unknown whether the PPAR activating effects of parabens and the antagonistic effects of butylparaben and prochloraz may be able to interfere with the activity of the endogenous ligands at physiologically relevant concentrations.

Our rat study showed no alteration of fetal testosterone production or anogenital distance by butylparaben, PFOA or rosiglitazone at the applied doses (Table 5 and Appendix Y). This study was not designed for studying whether PPARs are involved or not in chemically induced reduction of testosterone. If the compounds had been affecting fetal testosterone levels, this effect could be caused by interactions with other factors than PPARs. On the other hand, it cannot be excluded that PPAR interactions indeed do lead to reduced testosterone production, despite the lack of effects on testicular testosterone production of the applied doses of PFOA, butylparaben and rosiglitazone in the current study.

When comparing the potencies of the chemicals on PPAR transactivation *in vitro*, it becomes clear that much higher concentrations of PFOA and butylparaben are required for a certain response compared with MEHP in this assay (Appendix Y). Thus, the lack of effect *in vivo* may not be surprising, as the doses of chemicals applied in the rat studies are much lower for PFOA (20 mg/kg bw/day) and butylparaben (100 mg/kg bw/day) than the dose of DEHP (300 mg/kg bw/day), which reduced fetal testicular testosterone levels in our previous study (Paper IV). Rosiglitazone is more potent than MEHP with respect to PPAR γ transactivation *in vitro*, but still there is quite a gap between the applied dose of 1 mg/kg bw/day of rosiglitazone and the 300 mg/kg bw/day of DEHP required to see an effect on testosterone levels *in vivo*. For all the test compounds in the current study, however, higher doses than the applied were expected to be teratogenic or toxic to the dam according to studies described in the literature. Further studies may reveal whether higher doses of these compounds or other routes of administration will lead to antiandrogenic effects in fetal rats. Additionally, studies on distribution and metabolism of the compounds may reveal differences between fetal and adult exposure and metabolism, explaining why studies on other age groups of rats have revealed effects on testosterone levels at the applied doses.

As mentioned in relation to the phthalates, *in vitro* studies may be more adequate for determining whether the testosterone-reducing effect of these chemicals is related to their interaction with PPARs. If there is indeed a causal relation between PPAR activation and inhibition of testosterone production, reporter gene assays as those described here may be adequate for screening chemicals for PPAR activation and may thereby reveal other chemicals with potentially harmful effects adverse effects.

6.4.2 Prochloraz *in vivo* studies

The studies on the fungicide prochloraz presented in Paper VI are included as an example of an antiandrogen with a different mode of action compared to phthalates but with qualitatively rather similar effects. Prochloraz belongs to a group of widely used fungicides, the azoles, which may be suspected to have similar actions as prochloraz, but have not yet been thoroughly examined for endocrine effects. Prochloraz is presently being evaluated for classification as a reproductive toxicant due to its antiandrogenic effects. As mentioned, this compound is both an antagonist of the androgen receptor and reduces fetal testosterone production in rats²⁹. This induces reduced anogenital distance, increased nipple retention and altered behaviour of male rats exposed *in utero*³⁰. The impact of prochloraz on factors involved in steroidogenesis was examined by similar methods as the studies on DEHP and DIBP. Although prochloraz reduced testosterone production significantly in fetal testes similarly to the phthalates, prochloraz-exposed testes showed no alterations in expression of the examined genes, although immunohistochemical studies revealed altered protein expression of some of these factors.

The observed increase of testicular progesterone level indicates that prochloraz inhibits CYP17 activity, but the current immunohistochemical studies revealed an increased CYP17 expression (Paper VI). This indicates that the expression of CYP17 may be regulated by feedback mechanisms increasing CYP17 expression in response to the impaired activity of the enzyme. We also observed a reduced expression of 17 β HSD type 10 in Leydig cells of prochloraz exposed fetuses. 17 β HSD type 10 is highly expressed in fetal Leydig cells and

involved in the conversion of 3 α -androstenediol into dihydrotestosterone, which is an androgen with high affinity to the AR ⁸⁷. The reduced expression in prochloraz-exposed fetal testes may indicate yet another pathway leading to low androgen levels and in turn to the antiandrogenic effects observed with prochloraz exposure. Alternatively, 17 β HSD type 10 may be activated as a reaction to the low testosterone levels and/or impaired androgen function. The observed low expression of the protein may be associated with this increase in enzyme activity. Whether the low expression of 17 β HSD type 10 is an effect or a cause of the low testosterone levels remains unclear, but enzyme activity measurements would be relevant in order to reveal which of these hypotheses is true. Further studies may reveal whether reduced expression of this enzyme in fetal testes may be generally involved in the antiandrogenic effects and/or reduced testosterone production induced by other chemicals than prochloraz.

6.5 Dose-response and mixture studies - implications for risk assessment

The described studies on the mechanisms of action of endocrine disruptors are important in order to be able to detect other potential endocrine disruptors affecting the same targets. Furthermore, mechanistic knowledge may be important for determining whether the observed effects are relevant to humans and thus whether human exposure to the compound may pose a risk. Risk assessment is also based on knowledge on the dose levels at which adverse effects are observed in animal studies in comparison to the human exposure levels of the compound. Some vulnerable human population groups may be at risk of being exposed to phthalates at levels close to the dose levels inducing adverse effects in rat studies, as will be discussed in the following. Furthermore, as several phthalates and other compounds have comparable effects, there may be an increased risk with mixed exposure to all these compounds.

6.5.1 DEHP dose-response studies

The effects of gestational exposure to 0, 10, 30, 100 and 300 mg/kg bw/day of DEHP were examined in fetal rat testes at GD 21 (Paper IV). The studied endpoints seemed to be affected at different dose levels. The most sensitive endpoints were changes in gonocyte location and morphology as well as changes in mRNA levels of the steroidogenic regulator *StAR*, as the effects on these parameters were statistically significant at 100 mg/kg bw/day. Testicular testosterone levels and -production were reduced only in the highest dose group. However, for some endpoints such as testicular testosterone production and expression of other genes, changes seemed to be present at 30 and 100 mg/kg bw/day, but were not statistically significant with the selected number of animals. *PBR* and *StAR* mRNA levels were slightly, but not significantly, reduced already from 10 mg/kg bw/day of DEHP. Thus, if an increased number of litters had been used, statistically significant effects might have been revealed at lower dose levels. However, it can be difficult to interpret whether e.g. changes in gene expression at low dose levels are directly related to adverse effects and thereby relevant to risk assessment.

Risk assessment is generally based on dose-response studies examining endpoints that can be directly related to potentially adverse effects in humans. When rats were exposed to DEHP in gestation as well as in lactation, we observed adverse reproductive effects in adulthood such as small testes and epididymides and malformed secondary sexual organs at 300 and 750 mg/kg bw/day of DEHP (Paper III). Sperm quality was affected in a few animals in both dose groups. As no other doses were applied in that study, we could not determine a no-observed effect level (NOEL) applicable for risk assessment.

Andrade et al. (2006) examined adult Wistar rats exposed to DEHP in utero and via lactation. In that study, daily sperm production was reduced at doses from 15 mg/kg bw/day, while cryptorchidism was induced already at 5 mg/kg bw/day⁸⁸. In comparison, Gray et al. (2006) have reported abnormalities of male reproductive organs at doses from 11 mg/kg bw per day of DEHP (unpublished work reviewed by the same authors in ²⁸). The most recent risk assessment report by the European Chemical Bureau has agreed on a tolerable daily intake (TDI) of 48 µg/kg bw/day for DEHP based on a NOAEL of 4.8 mg/kg bw/day⁸⁹. This NOAEL was determined in a two-generation reproductive study showing reduced size of testes, epididymides and seminal vesicles and minimal testis atrophy at dose levels above 300 ppm corresponding to 4.8 mg/kg bw/day⁹⁰. Collectively, the NOAELs and LOAELs determined in these studies appear to be comparable. As some of the adverse effects are assumed to be caused by the low fetal testosterone level, it is remarkable that the adverse effects observed in the adults are actually found at lower dose levels than those reducing testosterone levels in our study.

In our combination study of DEHP and DINP, 750 mg/kg bw/day DINP induced slightly milder effects than 300 mg/kg bw/day of DEHP on fetal testicular testosterone levels and histopathology in our studies, indicating a lower potency of DINP than DEHP (Paper I and Table 4). This is also reflected in the risk assessment of these compounds, as DEHP is more strictly regulated than DINP regarding e.g. use in toys. Currently, DINP is not classified as toxic due to a relatively high NOAEL of 88 mg/kg bw/day based on adverse hepatic effects, but the EU scientific committee has recently suggested to use instead a NOAEL of 15 mg/kg bw/day giving a TDI of 150 µg/kg bw/day, which would lead to classification as a potential health hazard with prolonged exposure⁹¹. In contrast, our study on DIBP showed that DIBP is at least as potent as DEHP and DBP in reducing testosterone levels (Paper V), but DIBP is currently not classified, whereas DEHP and DBP are both classified as reproductive toxicants⁹².

6.5.2 Comparison to human exposure levels

These TDIs based on effects in animals studies can be used for comparison to human exposure levels and thereby assessment of risk. For phthalates, relatively high exposure levels have led to concern about the abundant use. Generally, mean exposure levels of adult humans are estimated to be in the range of 1 to 8 µg/kg bw/day, while maximal or 95th percentile exposure have been estimated to be in the range of 5 to 26 µg/kg bw/day²⁵. Another report determined an exposure level for DEHP by multiple pathways for consumers of 12 µg/kg bw/day for adults and 233 µg/kg bw/day for children⁸⁹. A recent study based on urinary metabolite levels has determined an exposure level of up to 74 µg/kg bw/day of DEHP (median 2 µg/kg bw/day) in a US study population of pregnant women⁹³.

Children are exposed to even higher levels of DEHP per kg body weight. Exposure levels of human infants are estimated to be in the range of 2 to 26 µg/kg bw/day with maximal or 95th percentile exposure levels between 16 and 285 µg/kg bw/day ²⁵. A recent report by the Danish Environmental Protection Agency (2006) on chemicals in consumer products reported a typical intake of 10-20 µg/kg bw/day of DEHP for children compared with a worst-case scenario of 50-250 µg/kg bw/day of DEHP for a very exposed child playing on a PVC floor. This indicates a narrow safety factor for DEHP for the highly exposed children, even without considering the concomitant exposure to other phthalates ⁹⁴. Collectively, maternal and infant exposure levels are close to or even exceed the TDI of 48 µg/kg bw/day ⁸⁹. Based on the high exposure levels, the US National Toxicology Program Centre for the Evaluation of Risks to Human Reproduction have expressed concern for effects of DEHP exposure on development of the male reproductive tract for infants ⁹⁵.

Due to high levels of DEHP in medical products, infants in neonatal intensive care are among the most exposed population groups. Infants in neonatal intensive care units have been estimated to have a median daily DEHP exposure of 42 µg/kg bw/day with 95th percentile at 1780 µg/kg bw/day ⁹⁶. These infants are thus exposed to levels near the TDI with some infants exceeding TDI several fold during a sensitive period of susceptibility. Additionally, adults receiving medical treatment can be exposed to very high levels of phthalates as certain tablets are coated with DBP. If these tablets are given to pregnant women, this may pose a risk to their unborn children. No studies on correlations between high neonatal or maternal phthalate exposure and reproductive function later in life have yet been reported.

This uncertainty factor of 100 between the NOELs and the tolerable daily intake is aimed at taking into account the interpersonal differences in sensitivity to chemical exposure and species differences in metabolic rate and sensitivity between rats and humans. Humans are known to have a more vulnerable reproductive system than rats, and minor defects of sperm production or function in men may render them infertile, whereas rats can reproduce even with large reductions of sperm production or quality ⁹⁷.

6.5.3 Human relevance of phthalate effects

It is remarkable that some of the phthalates, which were found to be associated with symptoms of the testicular dysgenesis syndrome in human studies ^{10,11}, do not induce adverse reproductive effects in rats. High urinary concentrations of metabolites of DBP, DIBP, DEP and dibenzyl phthalate were associated with reduced anogenital distance in boys ¹⁰, and high levels of monoester metabolites of DBP, DINP, DEP and DMP in mothers' breast milk were correlated with altered blood levels of reproductive hormone levels in their infant sons ¹¹. However, DEP and DMP do not affect reproductive development in male rats ¹⁷. This could indicate that the effects observed in the human studies are merely statistical coincidences. However, it is also possible that species differences lead to different mechanisms of action or different vulnerability to phthalates in humans compared to rats. An alternative explanation is that the levels of the primary monoester metabolites of DEP and DMP are markers of general phthalate exposure.

A comparison of measurements on maximal MEHP concentrations in blood in different species exposed to DEHP revealed that rats, marmosets and humans had a similar maximum MEHP concentration despite a 50

times larger dose given to rats and marmosets than humans¹⁴. This points to rather high “internal doses levels” in humans compared to rats even with low exposure levels. Additionally, the total MEHP exposure determined as the area under the concentration-time curves was several fold higher in humans despite the lower dose¹⁴. These species differences indicate an increased risk of phthalate toxicity in humans compared to rats or marmosets.

On the other hand, other species differences may imply that the mechanisms leading to adverse effects of phthalates in rats are not relevant to humans. Studies in marmosets have shown no reproductive effects of DEHP exposure of young and adult animals^{98;99}. On the other hand, boars prepubertally exposed to 50 mg DEHP/kg bw twice weekly had elevated testosterone levels and boars exposed to 300 mg DEHP/kg bw three times weekly had slight effects on sperm quality later in life^{100;101}. Rabbit studies have highlighted the importance of performing studies on perinatal phthalate exposure, as rabbits exposed to 400 mg/kg bw/day of DBP in utero had much more marked and permanent reproductive effects than rabbits exposed in adolescence or adulthood¹⁰². However, no studies on perinatal phthalate exposure of marmosets or boars have yet been reported why adverse reproductive effects of phthalates in these species cannot be excluded.

6.5.4 Mixture studies

The concept of using TDIs based on NOAELs takes only single chemicals into consideration. For the phthalates and many other groups of chemicals, it is highly relevant to consider the effects of combined exposure to compounds with similar mechanisms of action or similar effects in vivo. However, before that approach can be included in risk assessment, it is necessary to investigate how the compounds act in combination and to find appropriate prediction models for mixture effects.

DEHA is a non-phthalate plasticizer, which is used as a substitute for DEHP. The hypothesis that DEHA may modulate the effects of DEHP when these compounds are administered in combination was based on the fact that DEHP and DEHA share a common metabolite, 2-EH, when hydrolyzed in the gut to their monoesters, MEHP and mono(2-ethylhexyl) adipate (MEHA). The studies on combined exposure to DEHP and DEHA did not show any modulating effects of DEHA on the reproductive effects of DEHP, as exposure to DEHP + DEHA in combination had similar effects as exposure to DEHP alone (Paper I, II, III).

The lack of modulating effect of DEHA is in accordance with the general consideration that it is the monoester metabolite MEHP and not the other hydrolysis product 2-EH, which is responsible for the reproductive toxicity of DEHP. For example, rat pups exposed to MEHP exhibited testicular toxicity similar to that observed with DEHP, whereas no toxicity was present with 2-EH-exposure¹⁰³. As these studies did not include a group exposed to DEHA alone, they did not reveal whether DEHA (or 2-EH) affects e.g. fetal testicular testosterone production. However, a previous study by our group showed that perinatal DEHA exposure did not affect anogenital distance, nipple retention, sperm production or reproductive organ weights in rats at doses up to 800 mg/kg bw/day¹⁰⁴.

The combination study on DEHP and DINP showed increased effects of DEHP and DINP administered in combination compared to the effects of each compound alone (Paper I and poster in Appendix Y). As the study included only one dose level per compound, it could not be determined whether the effects were dose

additive, but clearly there was an accumulating effect. This supports the idea of recalculating TDIs by taking into account the contribution of other reproductive toxic phthalates.

Furthermore, exposure to other chemicals than phthalates with similar effects may also need to be considered. A study on rats exposed to BBP and the herbicide Linuron from GD 14-18 showed that these two compounds induced cumulative antiandrogenic effects compared to the effect of each compound alone¹⁰⁵. Animals in the BBP plus Linuron combination group had reduced fetal testosterone and progesterone levels, reduced neonatal and adult anogenital distance, increased infant and adult number of nipples/areolae and reduced reproductive tissue weight, while these effects were less marked in the animals exposed to the individual compounds. Interestingly, malformations of male external genitalia were only observed in the combination group and not for BBP or Linuron alone¹⁰⁵. Studies in rats on mixtures of chemicals with similar antiandrogenic actions performed by our group have demonstrated dose-additivity in rats. A combination of three AR antagonists induced marked feminization of male pups. In contrast, the same doses of each chemical alone did not give as marked effects (Hass et al. *in press*). A mixture of four chemicals with dissimilar mechanisms of action (AR antagonists, a 5 α -reductase inhibitor and DEHP) also induced higher levels of antiandrogenic effects than expected for each compound alone (unpublished). No mixture studies on different types of phthalates have been published previously to those included in this thesis (Paper I).

6.5.5 Implications for risk assessment

As long as it is unknown how many chemicals have similar effects or similar mode of action and whether they contribute to the total “antiandrogenic load” to humans, it is difficult to approach this problem in risk assessment. Instead, it may be reconsidered whether it is at all relevant to use the concept of a threshold dose for single chemicals below which no effects can be anticipated. In vivo as well as in vitro studies have shown that combined exposure to endocrine disrupting compounds at doses or concentrations below no-observed effect levels or no-observed effect concentrations can induce statistically significant effects when administered in combination^{106;107} (+ Hass et al. *in press*, Metzdorff et al., submitted). An alternative to the use of TDIs based on NOAELs for single chemicals could be the determination of a TDI for a group of chemicals with similar effects. For single chemicals, TDIs could be determined as a weighted fraction of the TDI for the group. However, these alternatives require added knowledge on the antiandrogenicity of a multitude of chemicals.

At present, the possibilities of detecting antiandrogenic endocrine disrupters using the current OECD regulatory guidelines are limited. The current studies showed reductions of the AGD at GD 21 or PND 3 for the chemicals that reduced fetal testicular testosterone levels, but not for the chemicals not affecting fetal testosterone. As the AGD is also reduced by antiandrogenic compounds with different mechanisms of action, measurement of AGD would be highly relevant as a standard endpoint in test guidelines in which animals are exposed in late gestation. Recent updates of the OECD two-generation test guideline studies do include the assessment of AGD in the second, but not the first, generation of rat offspring exposed to test chemicals, and only if triggered by findings in the first generation¹⁰⁸. The current studies further support that antiandrogenic chemicals alter AGD in fetal rats at GD 21, and it is therefore relevant to measure AGD at GD 21 in e.g. the

OECD guideline 414, which investigates prenatal developmental toxicity following chemical exposure from GD 7 to 21 similarly to our studies.

7 Conclusions and summary

In summary, the studies described in Paper I to VII have elaborated on a number of topics related to chemically induced reduction of fetal testosterone production. New knowledge revealed in the studies is listed here together with comments on the perspectives of these findings:

Phthalates

- DINP and DIBP have similar effects on fetal testicular development and testosterone levels as the known reproductive toxicant DEHP. This indicates a similar mechanism of action and points to a similar adverse effect pattern for these phthalates. There is thus a reason for concern for use of DINP and DIBP as substitutes for DEHP or DBP, and alternative substitutes need to be developed. Furthermore, the observation of an accumulating effect when exposing rats to a mixture of DEHP and DINP indicates that risk assessment should take into account the collective contribution of exposure to several reproductive toxic phthalates.
- The studies have elaborated on the temporal development of the testicular histopathological effects of DEHP, supporting the findings described for DBP by other investigators.
- DEHA is a non-phthalate plasticizer, which is used as a substitute for DEHP. The hypothesis that DEHA may modulate the effects of DEHP when these compounds are administered in combination was based on the fact that DEHP and DEHA share a common metabolite, 2-EH, when hydrolyzed in the gut to their monoesters, MEHP and MEHA. The studies showed no modulating effects of DEHA on the reproductive effects of DEHP.
- New insights have been made regarding phthalate mechanism of action. Reduced levels of factors involved in steroid synthesis cause the testosterone-reducing effect of DEHP and DIBP. This is similar to the alterations described for DBP by other authors. Based on literature studies and indications from in vivo studies, it is suggested that the phthalate mechanism of action may involve the nuclear receptors SF1, PPAR γ and PPAR α .
- As the evidence for involvement of PPARs or other nuclear receptors in the mechanism of action of phthalates is rather weak, further studies are needed. In particular, cell-based studies may be able to reveal whether PPAR α - or PPAR γ -activating or –inhibiting compounds affect steroid synthesis. If these receptors are indeed involved in the testosterone-reducing effects of phthalates, reporter gene assays such as those employed in the current studies may be valuable tools for screening chemicals for similar mode of action as phthalates and may thus reveal other chemicals with potentially harmful effects. In an in vitro screening battery testing the interaction of chemicals with e.g. the AR, estrogen receptor and thyroid receptor it would be relevant to include test of PPAR γ and PPAR α interaction.

Prochloraz

- Immunohistochemical studies on expression levels of factors involved in steroid synthesis elaborated on the mode of action of prochloraz, a fungicide that both acts as an antagonist to the androgen receptor and reduces fetal testosterone production. Reduced testosterone levels following prochloraz exposure is in part due to an inhibition of CYP17, a central enzyme in steroidogenesis. The observation of increased immunostaining intensity for CYP17 in Leydig cells of prochloraz-exposed fetuses may be a response to the inhibited steroid synthesis and emphasizes the importance of CYP17 in the mode of action of this compound.
- The expression of 17 β HSD type 10 was reduced in Leydig cells of prochloraz exposed fetuses. As this enzyme counteracts the degradation of testosterone and promotes dihydrotestosterone production, the lowered expression points to yet another mechanism leading to the observed antiandrogenic effects. Alternatively, the reduced expression may be a due to increased activity of the enzyme as a compensatory action towards the antiandrogenic effect of prochloraz. Enzyme activity measurements would be relevant in order to reveal whether the low expression of 17 β HSD type 10 is an effect or a cause of the low testosterone levels.
- A number of structurally comparable fungicides are abundantly used at present although their possible endocrine effects have not been determined. Further studies may reveal whether some of these compounds also act as antiandrogens.

Parabens

- Human exposure to parabens via personal care products and cosmetics is relatively high and knowledge on their possible adverse effects is lacking. Certain studies have shown that some of the parabens are estrogenic and are able to reduce testosterone levels and semen production of rats. Our preliminary study did not reveal any reduction of anogenital distance or testosterone levels in male fetal rats exposed to 100 mg/kg bw/day of butylparaben via oral dosing of the dam. Further studies are needed to determine whether higher doses of parabens or other routes of exposure may induce antiandrogenic effects in rats.
- We examined whether parabens are able to activate or antagonize the activation of PPAR α or PPAR γ in a reporter gene assay. Butylparaben and propylparaben were shown to be weak agonists as well as weak antagonists of PPAR α and PPAR γ in vitro. Further studies may reveal whether the interaction of parabens with PPARs can interfere with normal physiological processes in testes or other tissues.

Risk assessment of endocrine disruptors

- At present, regulatory test guidelines for chemical safety are unable to detect all types of endocrine disruption. The analysis of chemically induced changes in fetal testosterone levels seems to be highly relevant, because low testosterone in late gestation is associated with adverse effects later in life. A reduced anogenital distance (AGD) around the day of birth is a typical sign of antiandrogenic effects of chemicals, why this parameter would be relevant to include in future test guidelines.
- Prochloraz is an example of a compound with comparable antiandrogenic effects to the phthalates but dissimilar mechanism of action. It is plausible that mixtures of chemicals with similar effects but

dissimilar mechanisms of action lead to more severe effects than observed for each chemical alone. If future testing of chemicals reveals that several compounds interfere with e.g. fetal testosterone production by various mechanisms, the combined effect of exposure to even low doses of these chemicals may give rise to concern.

- It is presently unclear whether human exposure to endocrine disrupting chemicals is related to the increasing incidence of male reproductive disorders. As certain groups of infants have been exposed to extremely high levels of phthalates during treatment in neonatal intensive care units, future studies giving information on their reproductive function later in life may be valuable for the evaluation of the risk associated with perinatal phthalate exposure of humans.
- It is not evident whether phthalates or other compounds interfering with fetal testosterone production or –function are related to the testicular dysgenesis syndrome observed in humans. Each chemical alone may be present in human tissues at too low levels to cause any adverse reproductive effects. However, there is a reason for concern that several chemicals have antiandrogenic effects and that the increasing incidence of male reproductive dysfunction is due to combined exposure to antiandrogens. It is therefore highly relevant to be able to detect chemicals with antiandrogenic effects by use of adequate test guidelines and subsequently to be able to reduce human exposure to antiandrogens by regulatory initiatives.

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Appendix X – Published papers I to VI

- I. Borch J, Ladefoged O, Hass U, Vinggaard AM. Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats. *Reprod Toxicol.* 2004 Jan-Feb;18(1):53-61.
- II. Borch J, Dalgaard M, Ladefoged O. Early testicular effects in rats perinatally exposed to DEHP in combination with DEHA--apoptosis assessment and immunohistochemical studies. *Reprod Toxicol.* 2005 Mar-Apr;19(4):517-25.
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- V. Borch J, Axelstad M, Vinggaard AM, Dalgaard M. Diisobutyl phthalate has comparable antiandrogenic effects to di-n-butyl phthalate in fetal rat testis. *Toxicol Lett.* 2006 Jun 1;163(3):183-90.
- VI. Laier P, Metzдорff SB, Borch J, Hagen ML, Hass U, Christiansen S, Axelstad M, Kledal T, Dalgaard M, McKinnell C, Brokken LJ, Vinggaard AM. Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz. *Toxicol Appl Pharmacol.* 2006 Jun 1;213(2):160-71.

Corrections:

Paper I, Fig. 4A: Anogenital distance is indicated in mm on the y-axis, but these values are in fact arbitrary units, which must be multiplied by 0,165 in order to get the actual anogenital distance.

Paper II, Table I: Presence of chords with enlarged diameter: 0% of animals (0% of litters) affected in the control group, not 100% of litters.

Paper VI, Fig. 2B: Testosterone and progesterone was not measured in testes, but in the media of incubated testes.

Paper V: Animals & dosing: 32 mated rats were used in four blocks of eight, and not 24 as indicated.

Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats

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Abstract

The plasticizer di(2-ethylhexyl)phthalate (DEHP) exhibits antiandrogenic effects in perinatally exposed male rats. Di(2-ethylhexyl) adipate (DEHA) and diisononyl phthalate (DINP) are currently being evaluated as potential substitutes for DEHP, but similarities in structure and metabolism of DEHP with DEHA and DINP have led to the hypothesis that similarities in action may also exist. Pregnant Wistar rats were gavaged during gestation and lactation with vehicle, DEHP (300 or 750 mg/kg bodyweight per day), DINP (750 mg/kg bodyweight per day), DEHP (750 mg/kg bodyweight per day) in combination with DEHA (400 mg/kg bodyweight per day), or DEHP (300 mg/kg bodyweight per day) in combination with DINP (750 mg/kg bodyweight per day). DINP and DEHP were both shown to reduce testicular testosterone production *ex vivo* and testosterone levels in testes and plasma of male fetuses at gestation day 21, indicating a similar mechanism of action for DINP and DEHP. Additionally, plasma LH levels in male fetuses were elevated. Neonatal anogenital distance was reduced and the number of nipples at postnatal day 13 increased in DEHP-exposed male offspring. Serum inhibin B levels were significantly reduced in DEHP-exposed prepubertal male offspring, and in a few adult males. No modulating effects of DEHA on the endocrine effects of DEHP were detected, but a tendency towards an accumulating effect of DEHP and DINP in combination on suppression of testosterone synthesis was seen.

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Keywords: DEHP; DEHA; DINP; Phthalate; Antiandrogenic; Testosterone; Inhibin; Rat

1. Introduction

The plasticizer di(2-ethylhexyl)phthalate (DEHP) has been recognized as an endocrine disrupter inducing adverse effects in androgen responsive tissue following perinatal exposure of male rats [1]. Di(2-ethylhexyl) adipate (DEHA) and diisononyl phthalate (DINP) are currently being evaluated as potential substitutions for DEHP, but due to similarities in structure and metabolism of DEHP and DEHA, and of DEHP and DINP, it may be hypothesized that similarities in action may also exist. In the present studies, antiandrogenic effects in male rats perinatally exposed to DEHP alone or in combination with DEHA or DINP were investigated in late gestation, prepuberty or adulthood.

Antiandrogenic effects of phthalates have been suggested to be due to a reduced androgen availability in target organs causing malformations of male reproductive organs and low adult sperm counts [1,2]. Neither DEHP, di-*n*-butyl phthalate (DBP) or their monoester metabolites were able to an-

tagonize the androgen receptor [1,3]. Suppression of fetal androgen production by environmental chemicals has also been hypothesized of underlying the disorders comprising the ‘testicular dysgenesis syndrome’ in humans, i.e. testicular germ cell cancer, cryptorchidism, hypospadias and low sperm counts [4].

DEHP and DEHA are hydrolyzed in the gut to mono(2-ethylhexyl) phthalate (MEHP) and mono(2-ethylhexyl) adipate (MEHA), respectively, and have the metabolite 2-ethylhexanol (2-EH) in common (Fig. 1A). *In vivo* studies on MEHP have shown adverse effects on male reproductive development similar to those observed after DEHP exposure, effects which are not seen following 2-EH exposure alone [5,6]. On the other hand, 2-EH exhibits maternal and fetal toxicity when administered to pregnant rats [7]. Studies on DEHA have not shown any adverse effects on the reproductive system but rather developmental toxicity (prolonged gestation period, smaller pup size, high incidence of postnatal death) when administered to pregnant dams [8], effects resembling those observed with 2-EH. If 2-EH is also responsible for part of the effects of DEHP on the male reproductive system, DEHA may hypothetically augment DEHP-

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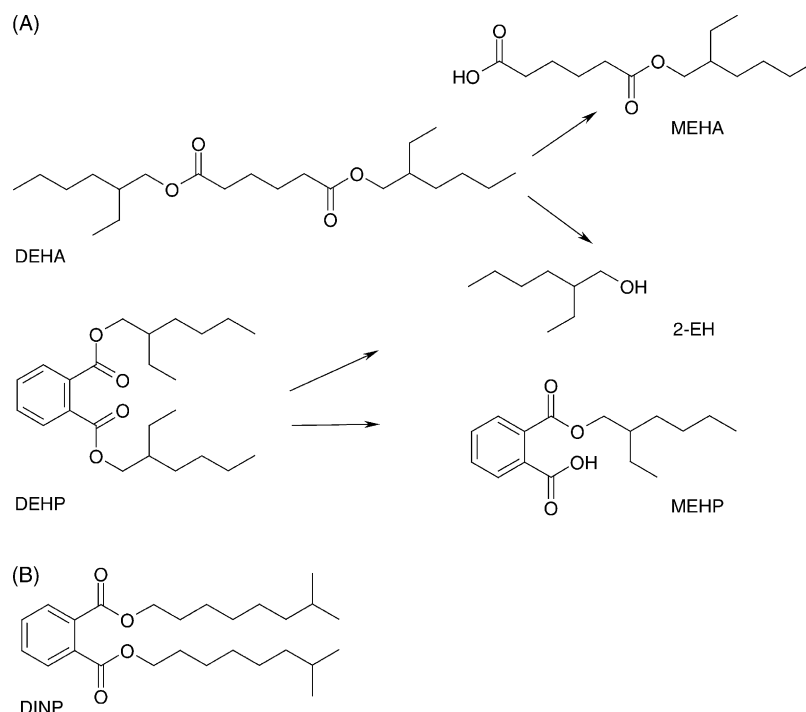


Fig. 1. (A) Metabolism of di(2-ethylhexyl) phthalate (DEHP) and di(2-ethylhexyl) adipate (DEHA) to 2-ethylhexanol (2-EH) and mono(2-ethylhexyl) phthalate (MEHP) or mono(2-ethylhexyl) adipate (MEHA) and (B) structure of diisononyl phthalate (DINP).

induced changes in male reproductive endpoints when the two compounds are administered in combination, even though DEHA does not produce these effects on its own.

Some studies on DINP (Fig. 1B) have shown effects on androgen responsive tissues (nipple retention, testes atrophy, epididymal agenesis) in males [2], but in other studies DINP was reported as having no adverse reproductive effects [9]. The malformations observed with perinatal DINP exposure point to a similar mechanism of action as DEHP, and modulating effects of DINP on DEHP-induced changes may be hypothesized when the compounds are administered together.

In the present studies, male offspring of dams exposed to DEHP, or DEHP in combination with DEHA were examined in late gestation, prepuberty and adulthood. Among the examined parameters were effects on androgen dependent development (anogenital distance and nipple retention), ex vivo testicular testosterone production, blood levels of testosterone, inhibin B, luteinizing hormone (LH), and follicle stimulating hormone (FSH). In addition, DEHP and DINP and a combination of the two were investigated for effects on fetal testosterone and LH levels.

2. Materials and methods

2.1. Test compounds

DEHP, di(2-ethylhexyl) phthalate, CAS no. 117-81-7, purity 99%, was from Aldrich 20,115-4. DEHA, di(2-ethylhe-

xyl) adipate, CAS no. 103-23-1, purity 99%, was from Aldrich 29,118-8. DINP, diisononyl phthalate, CAS no. 28553-12-0, purity >99%, was from Aldrich 37,666-3. The vehicle was peanut oil obtained from the Royal Veterinary Agriculture Pharmacy, Copenhagen, Denmark or from Sigma.

2.2. Animals and dosing

2.2.1. Experiment 1

This study was designed to investigate hormonal effects in male rat fetuses exposed to DEHP, DINP or a combination of DEHP and DINP. Thirty-two dams were randomized into four groups dosed with either vehicle (peanut oil), 300 mg DEHP/kg bodyweight per day, 750 mg DINP/kg bodyweight per day, or with 300 mg DEHP/kg bodyweight per day plus 750 mg DINP/kg bodyweight per day. Male fetuses were sacrificed on gestation day (GD) 21, and blood and testes were collected for hormone analyses. The dose of 750 mg DINP/kg bodyweight per day was the same as used by Gray et al. [2]. The dose of 300 mg DEHP/kg bodyweight per day was selected in order to obtain submaximal effects on hormone levels compared to the dose of 750 mg DEHP/kg bodyweight per day used in Experiment 2 and by Parks et al. [1]. Additionally, these doses were not expected to cause maternal toxicity when administered in combination.

2.2.2. Experiment 2

This study was designed to investigate effects of combined exposure to DEHP and DEHA in male rats in late

gestation and early postnatally. Forty-eight time-mated Wistar rats (Mol:WIST, SPF, Taconic M&B, Denmark) were housed in pairs and randomized into three groups with similar body weight distributions. The day following mating was designated GD 1, and postnatal day (PND) 1 was the day following birth. The animals were dosed with either vehicle (peanut oil), 750 mg DEHP/kg bodyweight per day, or 750 mg DEHP/kg bodyweight per day in combination with 400 mg DEHA/kg bodyweight per day from GD 7 to PND 17. On GD 21, eight randomly selected dams per group were anesthetized in CO₂/O₂ and decapitated, and fetuses were removed and decapitated. Blood and testes from male fetuses were collected for hormone analyses. The remaining eight dams per group were housed in individual cages at GD 21, and anogenital distance at PND 3 and nipple retention at PND 13 were assessed in all male offspring as previously described [8]. The dose of 750 mg DEHP/kg bodyweight per day was the same as in the study by Parks et al. [1]. The dose of 400 mg DEHA/kg bodyweight per day was selected as this was the highest dose not causing postnatal death of offspring or maternal toxicity in the study by Dalgaard et al. [8]. These doses were not expected to cause maternal toxicity when administered in combination.

2.2.3. Experiment 3

This study was designed to investigate effects in prepuberty and adulthood in male rats perinatally exposed to DEHP and DEHA in combination. Eighty time-mated Wistar rats were randomized into four groups receiving either 0, 300 or 750 mg DEHP/kg bodyweight per day, or 750 mg DEHP/kg bodyweight per day in combination with 400 mg DEHA/kg bodyweight per day from GD 7 to PND 17. Three randomly selected males per litter from 10 litters per group were sacrificed at PND 22, and further one or two males per litter were sacrificed at PND 190. Blood from all males, and testes from one male per litter at PND 190 were collected for hormone analyses. The doses were the same as in Experiment 2 but with an additional dose of 300 mg DEHP/kg bodyweight per day in order to investigate dose-effect relations.

2.3. Hormone analyses

2.3.1. Ex vivo testosterone production

The left testes from two randomly selected fetuses per litter were placed in 1 ml ice-cold Dulbecco's Modified Eagle Medium (DMEM)/F12 with 15 mM HEPES, 365 mg/l L-glutamine plus 0.1% bovine serum albumin. The testes were incubated at 34 °C for 3 h, were then placed on ice, centrifuged at 4000 × g for 10 min, and the supernatant was collected and stored at –80 °C until analysis of testosterone content.

2.3.2. Extraction of testicular testosterone

One testis from one randomly selected male per litter of fetal or adult rats was placed in an empty tube and imme-

diately frozen in liquid nitrogen and stored at –80 °C until analysis. Testosterone was extracted by two consecutive diethylether extraction procedures and stored at –80 °C until analysis.

2.3.3. Extraction of plasma and serum testosterone

Blood was centrifuged at 4000 × g for 10 min at 4 °C. Pools of plasma from fetuses and serum from 22- and 190-day-old males was kept at –80 °C until analysis of testosterone, LH, FSH or inhibin B content. Steroids were extracted from blood samples by solid-phase extraction using IST Isolute C18 SPE columns of 100 mg.

2.3.4. Hormone analyses

Testosterone content in incubation media, testes and blood was analyzed with a Delfia Testosterone Time-resolved fluoroimmunoassay (A050-201, PerkinElmer, Wallac Oy, Turku, Finland). Serum LH and FSH levels were analyzed at Turku University, Finland. Rat FSH immunoreactivity was determined by a two-site immunofluorometric assay recognizing both the β -subunit and the α -subunit of the FSH molecule [10]. Rat LH was measured using the time-resolved fluorimetric assay (Delfia, Wallac OY, Turku, Finland) as described by Haavisto et al. [11]. Inhibin B was measured using an Inhibin B ELISA assay (MCA1312KZZ, Oxford Bio-Innovation, Oxford, UK) recognizing both the β B-subunit and the α -subunit of inhibin B.

2.4. Statistics

Non-processed and logarithmically transformed data were examined for normal distribution and homogeneity of variance. Anogenital distance and nipple data were analyzed by ANOVA using litter means. Anogenital distance data were also analyzed by ANCOVA with body weight as a covariate. All other data were analyzed by ANOVA using single animal data nested within litter, which was the statistical unit. Litter was included in the analysis of variance as an independent, random factor (SAS Analyst: mixed procedure, SAS version 8, SAS Institute Inc, Cary, NC, USA). Dunnett's tests were performed to determine differences between treated and control group means. Additionally, interaction effects were determined on data from Experiment 1 in a factorial analysis with the factors DEHP and DINP at levels – or +. Calculation of Pearson's r was followed by a t -test of the hypothesis $r = 0$. Asterisks indicate a statistically significant difference compared to controls *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

3. Results

3.1. Fetal testosterone and LH levels, Experiment 1

In the DEHP + DINP combination study, statistically significant reductions in testicular testosterone production and in testicular testosterone content were observed in DEHP,

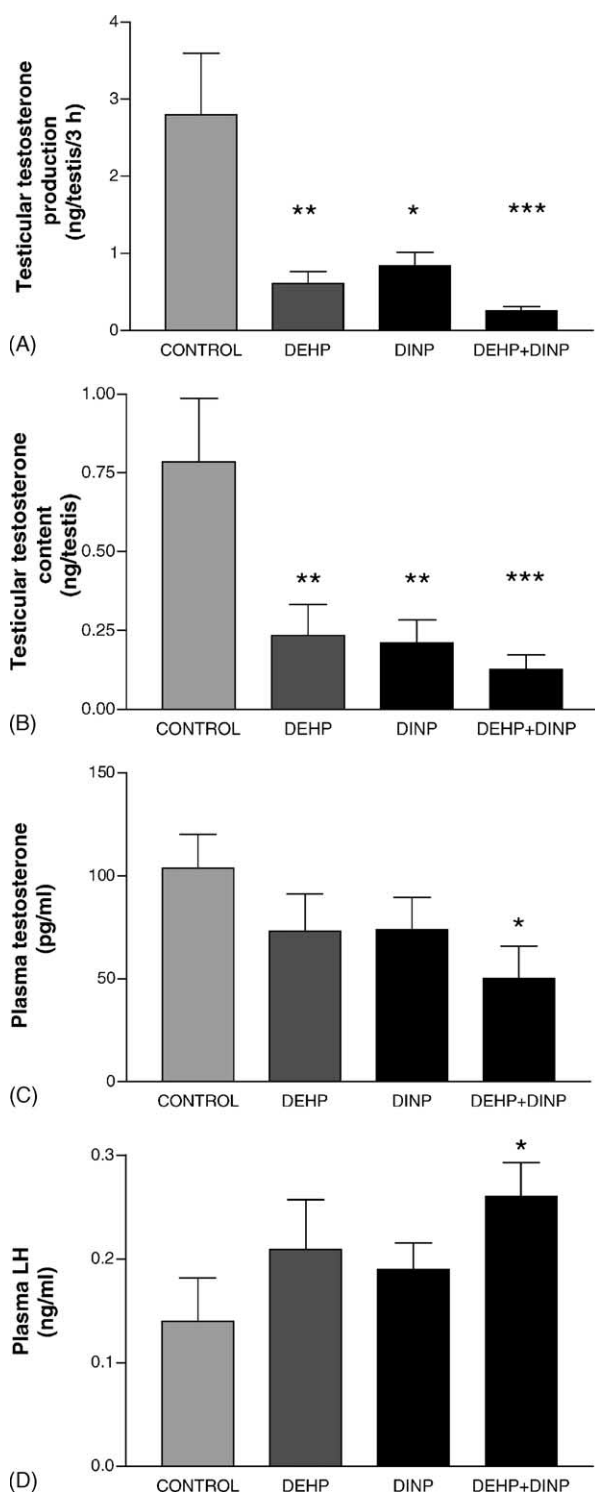


Fig. 2. Testicular testosterone production (A), testicular testosterone content (B), plasma testosterone (C) and plasma LH (D) in 21-day-old male rat fetuses exposed to vehicle, 300 mg DEHP/kg bodyweight per day, or 750 mg DINP/kg bodyweight per day or 300 mg DEHP/kg bodyweight per day in combination with 750 mg DINP/kg bodyweight per day from GD 7 to GD 21 (Experiment 1). Values are means \pm S.E.M. (A) Two testes from each of seven to eight litters per group, $n = 14$ –16 testes per group; (B) one to two testes from each of six to ten litters per group, $n = 6$ –11 testes per group; (C) pools of plasma from 1 to 2 litters, $n = 5$ –7 samples per group; (D) pools of plasma from 1 to 2 litters, $n = 3$ –7 samples per group. Asterisks indicate a statistically significant difference from controls * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

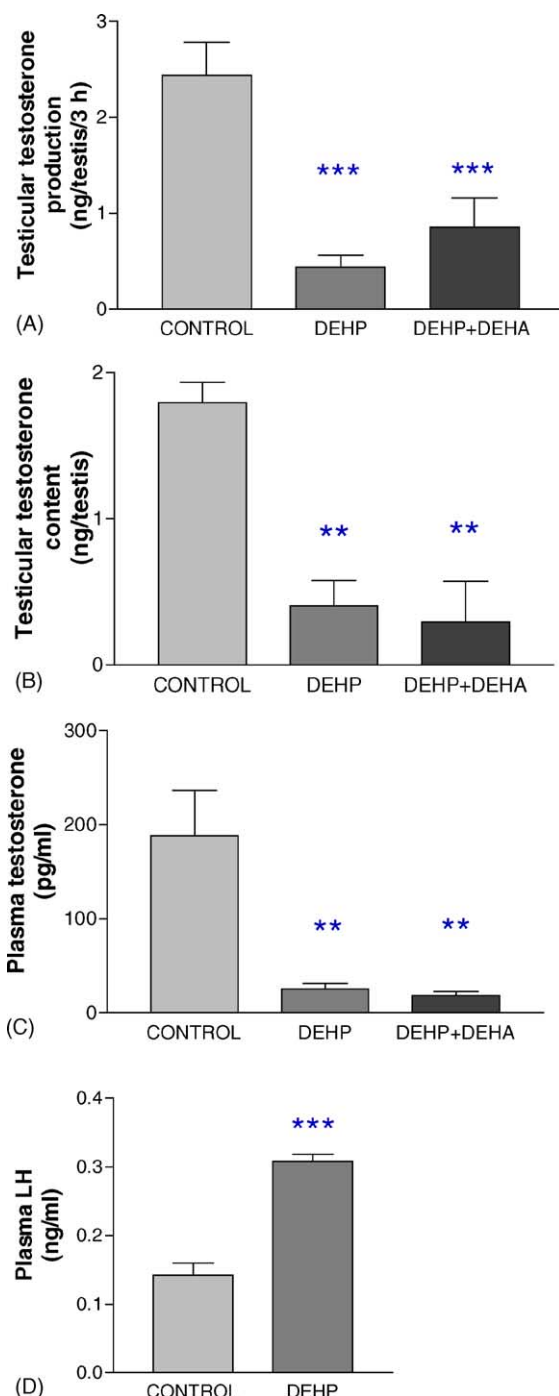


Fig. 3. Testicular testosterone production (A), testicular testosterone content (B), plasma testosterone (C) and plasma LH (D) in 21-day-old male rat fetuses exposed to vehicle, 750 mg DEHP/kg bodyweight per day, or 750 mg DEHP/kg bodyweight per day in combination with 400 mg DEHA/kg bodyweight per day from GD 7 to GD 21 (Experiment 2). Values are means \pm S.E.M. (A) Two testes from each of six to eight litters per group, $n = 14$ –19 testes per group; (B) one testis from each of six to eight litters per group, $n = 6$ –8 testes per group; (C) pools of plasma from one to two litters, $n = 2$ –6 samples per group; (D) pools of plasma from one to two litters, $n = 3$ –5 samples per group. Asterisks indicate a statistically significant difference from controls ** $P < 0.01$, *** $P < 0.001$.

DINP and DEHP + DINP treated animals compared to controls (Fig. 2A and B). A similar tendency was seen for plasma testosterone, although the reduction was only statistically significant for the DEHP + DINP combination group (Fig. 2C). Plasma LH levels were elevated, and again the difference was only statistically significant for the DEHP + DINP combination group compared to controls (Fig. 2D). No statistically significant differences in hormone levels were observed between the DEHP + DINP group and the groups receiving DEHP or DINP alone. A factorial statistical analysis revealed no statistically significant interaction between the effects of DEHP and DINP.

3.2. Fetal testosterone and LH levels, anogenital distance and nipples, Experiment 2

In Experiment 2, on fetal and early postnatal effects of DEHP + DEHA in combination, male offspring of DEHP-

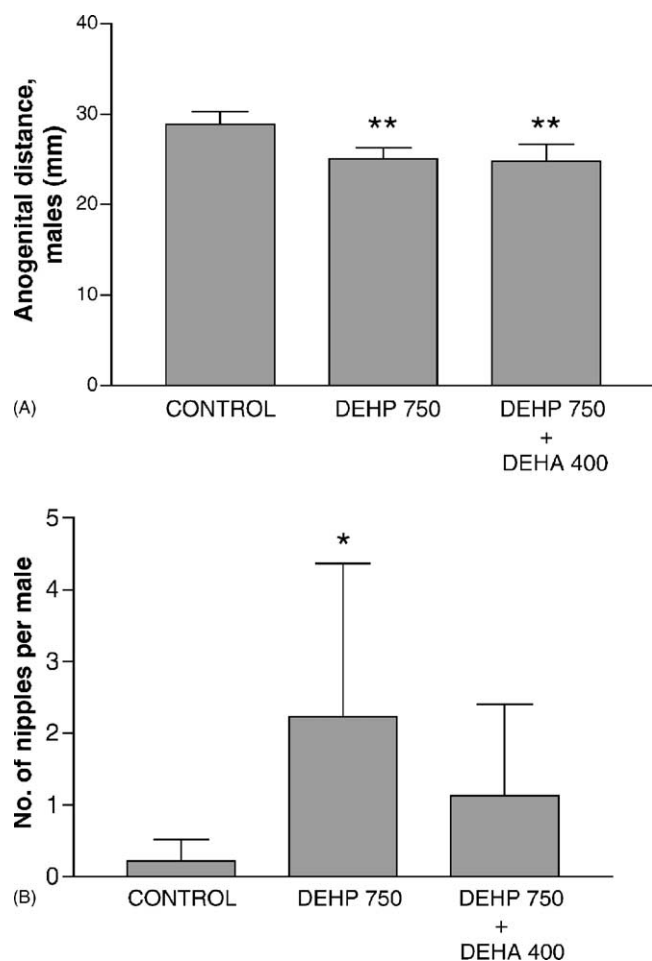


Fig. 4. Neonatal anogenital distance at PND 3 and number of nipples at PND 13 in male rats exposed to vehicle, 750 mg DEHP/kg bodyweight per day, or 750 mg DEHP/kg bodyweight per day in combination with 400 mg DEHA/kg bodyweight per day from GD 7 to GD 21 (Experiment 2). Values are means \pm S.D., $n = 35$ males from six to seven litters per group. Asterisks indicate a statistically significant difference from controls * $P < 0.05$, ** $P < 0.01$.

treated and DEHP + DEHA-treated rats showed a reduced ability to produce testosterone on GD 21. Testosterone production in testes incubated for 3 h ex vivo was statistically significantly reduced in the DEHP and DEHP + DEHA groups compared to controls (Fig. 3A). Similarly, statistically significant reductions in testicular testosterone content were observed in DEHP treated and DEHP + DEHA treated male fetuses compared to controls (Fig. 3B).

Testosterone levels in plasma from male fetuses were statistically significantly reduced in the DEHP and DEHP + DEHA groups compared to controls (Fig. 3C). In addition, a statistically significant increase in plasma LH content was measured in DEHP treated fetuses compared to controls (Fig. 3D). Due to limited amount of plasma from DEHP + DEHA treated fetuses, LH was not measured in that group.

Anogenital distance at PND 3 was significantly reduced in the DEHP-exposed males and in the males exposed to DEHP plus DEHA (Fig. 4A). The statistical analysis using birth weight as a covariate gave similar results and birth weight had no significant influence on the anogenital distance.

The number of nipples was significantly increased at PND 13 in males exposed to DEHP, but not in males exposed to DEHP plus DEHA (Fig. 4B). The effect of DEHP and DEHA in combination did not differ significantly from the effect seen with DEHP alone.

3.3. Testosterone, inhibin B, FSH and LH levels at PND 22 and 190 (Experiment 3)

In Experiment 3 on prepubertal and adult effects of DEHP + DEHA in combination, serum inhibin B levels were significantly reduced in 22-day-old males exposed to 750 mg DEHP/kg bodyweight per day compared to controls (Fig. 5A). The average inhibin B level in animals exposed to 300 mg DEHP/kg bodyweight per day appeared lower than in controls and higher than in animals exposed to 750 mg DEHP/kg bodyweight per day, indicating a dose-dependent effect. Inhibin B levels were similar in the group receiving 750 mg DEHP/kg bodyweight per day and in the group receiving DEHP in combination with DEHA. On PND 190, no differences in inhibin B levels between groups were observed (Fig. 6C). No correlation existed between inhibin B levels measured in males from the same litters on PND 22 and PND 190 (data not shown).

A tendency towards increased serum FSH levels with increasing DEHP dose was observed on PND 22 (Fig. 5B). However, no statistically significant differences between the four groups were detectable, likely due to the large variations between animals within each group.

Serum LH levels were not significantly different between groups on PND 22, but while LH levels varied only little within control litters, half the litters in the high-dose DEHP group had several-fold higher LH levels than controls (Fig. 5C). Five of the litters with the highest LH levels in the exposed groups were also the litters with the highest

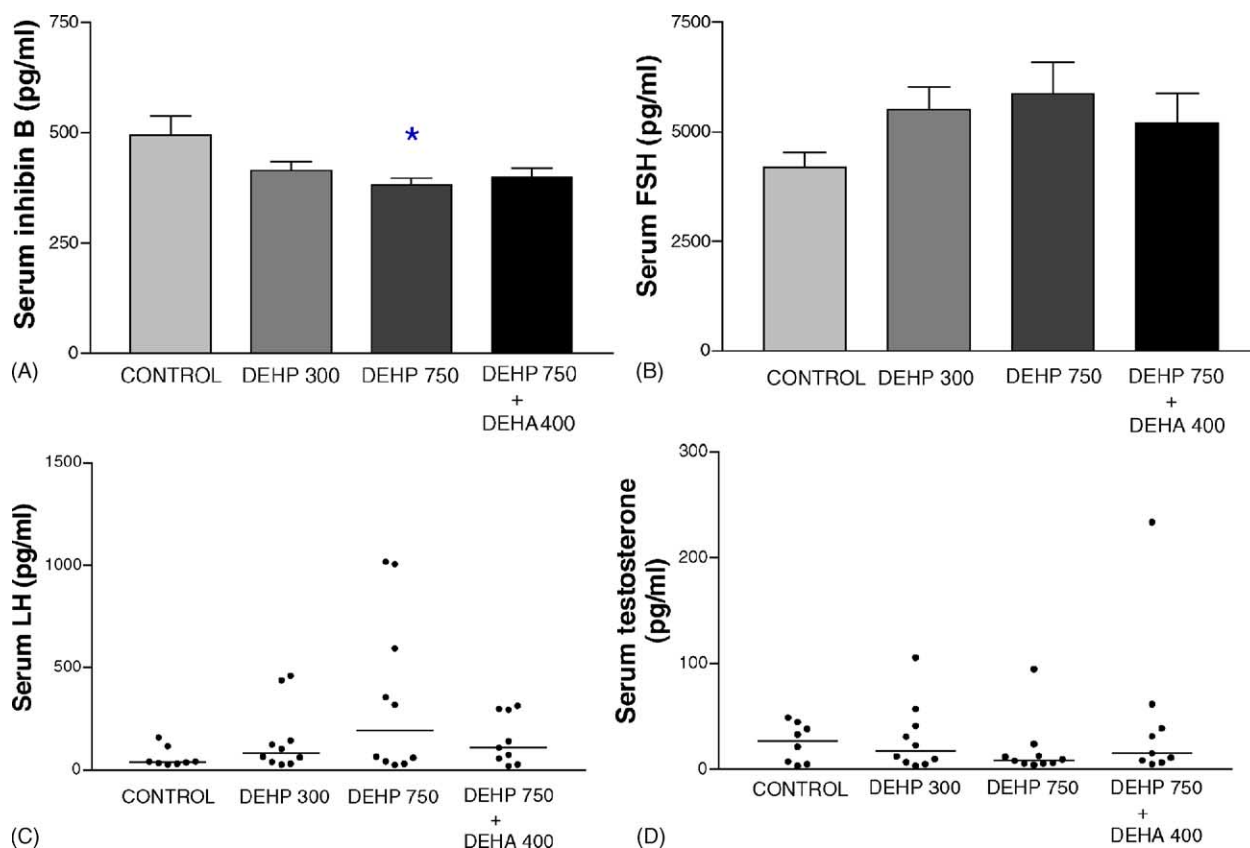


Fig. 5. Serum hormone levels at PND 22 in male rats exposed to either vehicle, 300 or 750 mg DEHP/kg bodyweight per day, or 750 mg DEHP/kg bodyweight per day in combination with 400 mg DEHA/kg bodyweight per day from GD 7 to PND 17 (Experiment 3). (A) serum inhibin B, (B) serum FSH, (C) serum LH, (D) serum testosterone. Values are means \pm S.E.M. (A and B), or single measurements (C and D) on serum pooled from three males per litter ($n = 8$ –10 samples per group), where lines designate median values. Asterisks indicate a statistically significant difference from controls $*P < 0.05$.

FSH levels. The litters with very high serum LH and FSH levels also had low serum inhibin B levels and high or normal serum testosterone levels. A correlation between serum LH and serum FSH (Pearson's $r = 0.61$, $P < 0.001$) and an inverse correlation between serum FSH and serum inhibin B (Pearson's $r = -0.51$, $P < 0.001$) were observed.

No significant differences in serum testosterone levels between groups were detectable on PND 22 (Fig. 5D). When considering serum testosterone levels and testicular testosterone content at PND 190, a pattern comparable to the reduced testosterone levels in gestation was seen, with the strongest tendency observed for the DEHP + DEHA combination group (Fig. 6A and B), although no statistically significant differences were found.

4. Discussion

4.1. Endocrine effects of DEHP and DINP

The study on gestational DINP exposure (Experiment 1) revealed for the first time an inhibitory effect on fetal testicular testosterone production as well as testicular testosterone content, and a tendency to reduced plasma

testosterone level. The observed reduction of ex vivo testosterone production, testicular testosterone content and plasma testosterone in 21-day-old fetuses receiving DEHP *in utero* confirmed previous findings by Parks et al. [1]. In agreement with the reported lower antiandrogenic potency of DINP than DEHP [2], 750 mg/kg bodyweight per day of DINP reduced testosterone to levels comparable to those observed with the lower dose of 300 mg/kg bodyweight per day of DEHP (Fig. 2A–C). Also in the DEHP + DEHA combination study (Experiment 2), testosterone levels in the DEHP group and the DEHP + DEHA group were reduced significantly compared to controls. Abnormalities of androgen-dependent tissues paralleled the reduction in testosterone levels: anogenital distance was significantly reduced and the number of nipples was increased in males exposed to DEHP. These effects have been observed in other studies of prenatal DEHP exposure [1,2], and are likely to be causally linked to the low testosterone levels. It appears that testosterone levels in fetuses are affected in more animals than develop abnormalities later on.

Concomitantly with the decrease in testosterone level, a significant increase in plasma LH was observed in males receiving 750 mg DEHP/kg bodyweight per day (Experiment 2), a finding not previously described in studies on prenatal

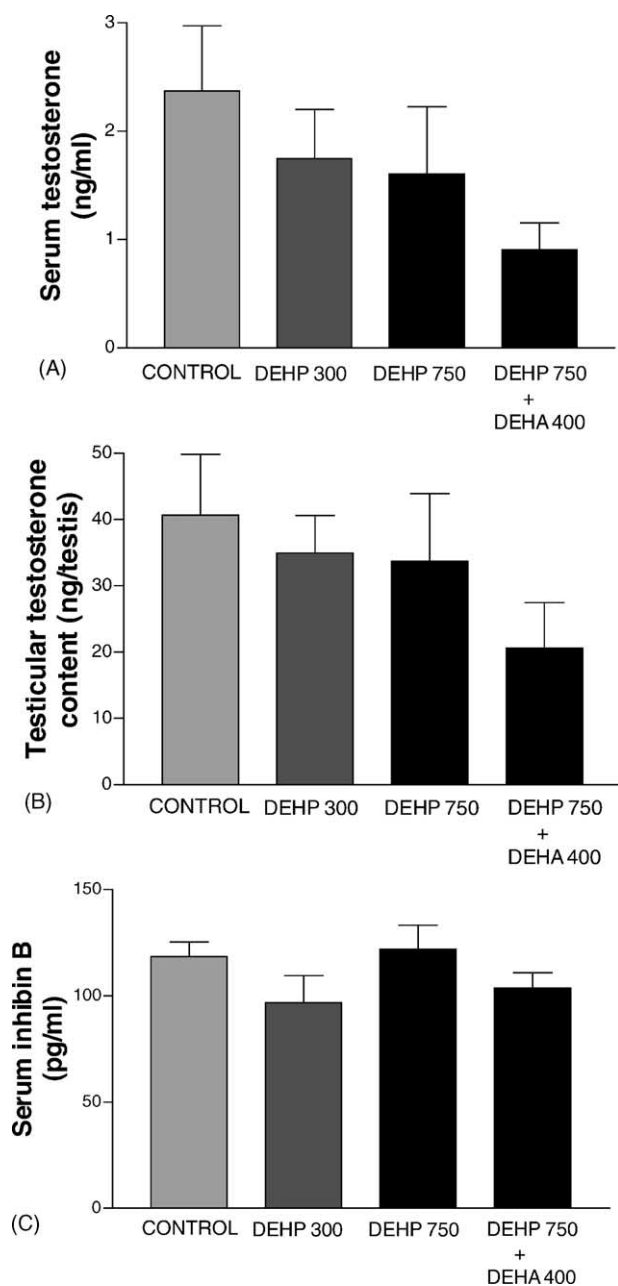


Fig. 6. Serum testosterone (A, $n = 13$ –16 males per group), testicular testosterone content (B, $n = 9$ –10 males per group) and serum inhibin B (C, $n = 10$ males per group) at PND 190 in male rats exposed to either vehicle, 300 or 750 mg DEHP/kg bodyweight per day, or 750 mg DEHP/kg bodyweight per day in combination with 400 mg DEHA/kg bodyweight per day from GD 7 to PND 17 (Experiment 3). Values are means \pm S.E.M.

phthalate exposure. A similar elevation in plasma LH was seen in the DINP study (Experiment 1), although the effect was only statistically significant for the DEHP + DINP combination group in that study. This observation indicates the presence of a functional feedback loop from the gonads to the hypothalamus and pituitary on GD 21 under the current experimental conditions. Plasma LH levels do not normally start rising until GD 21 [12], although Leydig cell

responsiveness to LH appears at GD 19. At this age plasma testosterone has already started peaking independently of LH action. Apparently, the increase in plasma LH was not sufficient to re-establish testosterone production at GD 21, indicating that the functional defect induced by DEHP may either be found within the Leydig cell, or that LH action has been modulated by other factors. Changes in the activity or expression of steroidogenic enzymes following phthalate exposure have been found in rat studies on fetal DBP exposure [13,14]. However, it is not evident whether such changes are due to direct interactions of phthalates with Leydig cells, or to indirect effects caused by phthalate interference with Sertoli cell function and production of paracrine factors acting on Leydig cells. Experiments on Leydig tumor cells indicate that MEHP may have direct effects on Leydig cell lipid metabolism and progesterone synthesis [15,16]. Previously, phthalate effects were ascribed mainly to interference with Sertoli cell function [17,18], but the current evidence suggests that also the Leydig cell population is targeted in the fetus.

At PND 22 (Experiment 3), serum inhibin B levels were significantly lower in the high-dose-DEHP and DEHP + DEHA exposed groups compared to controls, but serum testosterone, LH and FSH levels did not differ significantly between groups. A correlation between serum levels of LH and FSH for individual animals was observed at PND 22, in addition to an inverse correlation between serum levels of FSH and inhibin B. In a few exposed animals, a characteristic pattern of high serum levels of LH, FSH and testosterone concomitantly with low serum inhibin B levels was observed. Akingbemi et al. [19] have found the opposite effect: serum testosterone and LH levels were significantly lower on PND 21 and 35 in the offspring of rats treated with 100 mg DEHP/kg bodyweight per day from GD 12 to GD 21. In the offspring of rats exposed to similar doses during lactation as well as in utero (GD 12 to PND 21) serum testosterone was slightly reduced on PND 21 but not on PND 35 [19]. Due to the nature of pubertal development, endocrine effects at this age may indicate a time-shift in the developmental process, whereas the tendencies to effects on adult serum inhibin B and testosterone levels (Fig. 6A–C) may suggest long-term effects on testicular function. The significantly reduced serum inhibin B level in prepubertal DEHP-exposed males indicates a reduced Sertoli cell number or function, and is in accordance with the presumption that phthalates disrupt the FSH response, and that the FSH level in neonatal life determines Sertoli cell number [20]. Serum inhibin B levels and testes weights (Majken Dalgaard, personal communication) were unaltered in most of the DEHP exposed adults, indicating that recovery of Sertoli cell number or function had occurred since prepuberty in most animals. However, a group of five adult males had very low serum inhibin B levels (below 70 pg/ml), and three of these males were reported as having atrophic testes (Majken Dalgaard, personal communication). In addition to these effects in few animals, the tendency to reduced mean testos-

terone levels in serum and testes of adult males, mainly in the DEHP + DEHA combination group, may point to permanent functional defects also in males without testicular atrophy.

4.2. Effects of combined exposure

No modulating effect of DINP on the effects of DEHP can be inferred on the basis of these data, as fetal testosterone levels were not significantly lower with exposure to DINP in combination with DEHP than with DEHP or DINP alone. However, a tendency indicating an accumulating effect of DEHP and DINP in reducing steroidogenesis could be observed (Fig. 2A–C). Factorial statistics revealed no interaction effect, indicating that the effect of the combination was in agreement with what could be expected from addition of the DINP and the DEHP doses.

No statistically significant differences in endocrine effects were observed between rats exposed to DEHP and rats exposed to DEHP in combination with DEHA. A shortfall of these studies is the lack of a group receiving DEHA alone. However, as the effects in the exposed groups were quantitatively similar, it was concluded that DEHA does not modulate the effects of DEHP. If, on the other hand, the applied 750 mg DEHP/kg bodyweight per day suppresses fetal testosterone levels maximally, detection of further suppression by DEHA may be difficult. Selecting dose levels based on dose response curves will be necessary to fully establish whether combination effects may be seen. Previous studies have shown no effect of gestational and lactational DEHA exposure (up to 800 mg/kg bodyweight per day) on male reproductive development [8]. In that study, prepubertal and adult testicular testosterone content and prepubertal serum LH level were unaffected by DEHA, and no changes in anogenital distance, number of nipples, histopathology or weights of reproductive organs were observed. The lack of modulating effect of DEHA on the endocrine effects of DEHP in this study strengthens the conclusion that DEHA does not induce antiandrogenic effects. In the literature, no evidence for testicular toxicity of the metabolite 2-EH was found [7].

In summary, these studies reveal that DINP has a suppressing effect on fetal testosterone levels in prenatally exposed male rats similarly to the effect observed with DEHP. Correspondingly, fetal plasma LH levels were increased in DEHP-exposed males. As expected, neonatal anogenital distance was reduced and the number of nipples increased in DEHP-exposed males. The extensive endocrine effects of DEHP in late gestation were absent in prepuberty and adulthood, except for a reduced serum inhibin B level in 22-day-old males, and in a few adult males. Administration of DEHA or DINP in combination with DEHP did not significantly modulate the endocrine effects of DEHP, although a tendency to an accumulating effect in suppressing testosterone synthesis was seen in the DINP and DEHP combination study.

Acknowledgments

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Early testicular effects in rats perinatally exposed to DEHP in combination with DEHA—apoptosis assessment and immunohistochemical studies

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Abstract

This study aimed to characterize the effects of di(2-ethylhexyl) phthalate (DEHP) on the fetal rat testes and relate them to the effects seen in adults. Histopathological effects in fetal testes were examined with immunohistochemistry for anti-Müllerian hormone (AMH), 3 β -hydroxysteroid dehydrogenase, smooth muscle actin (SMA), proliferating cell nuclear antigen (PCNA), histone H3 and vimentin. Additionally, testicular apoptosis levels were assessed in fetal, prepubertal and adult rats. As the plasticizer di(2-ethylhexyl) adipate (DEHA) has similarities with DEHP in chemical structure and metabolism, we investigated if the testicular effects of DEHP were modulated by co-administration with DEHA.

Wistar rats were gavaged during gestation and lactation with vehicle, DEHP (300 or 750 mg/kg/day), or DEHP (750 mg/kg/day) in combination with DEHA (400 mg/kg/day), and male offspring were examined at gestation day (GD) 21, postnatal day (PND) 22, 26 and 190.

In fetal testes, Leydig cells were found in large clusters containing AMH positive Sertoli cells. At GD 21, seminiferous chords appeared enlarged with an apparently increased number of gonocytes. However, proliferation of gonocytes did not appear increased. A few animals had a high number of TUNEL positive apoptotic cells in degenerating seminiferous tubules at PND 22 and 190, whereas most exposed animals had low levels of germ cell apoptosis at GD 21, PND 22 or PND 26, as evaluated by DNA laddering, TUNEL staining, Caspase-3 immunohistochemistry and Caspase-3 activity measurement. No differences between DEHP and DEHP + DEHA exposed groups were observed.

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Keywords: Phthalate; Toxicity; Male reproduction; In utero; Programmed cell death; Pathology; Endocrine disrupters

1. Introduction

Phthalates are extensively used in consumer plastic products, and human exposure is considerable. Certain phthalates are known to induce adverse effects in androgen responsive tissues (cryptorchidism, hypospadias, decreased sperm counts, decreased weights of reproductive organs) following perinatal exposure of male laboratory animals [1–3]. Studies concerning pathogenesis of effects have been performed with dibutyl phthalate (DBP) [4–6]. The current study aims to test if similar early testicular effects are seen with di(2-ethylhexyl) phthalate (DEHP), which is among the phthalates

most abundantly found in the environment [7]. It is essential to evaluate the effects of perinatal exposure, as the perinatal period is particularly sensitive to hormonal influences and as infants are exposed to phthalates in considerable doses [7].

Following acute DEHP or MEHP treatment, germ cell apoptosis is induced in adult and prepubertal rat testes by disruption of interaction between Sertoli cells and germ cells [8,9]. Also reduction of the intratesticular testosterone level has been shown to induce germ cell apoptosis in adult or prepubertal rats [10,11]. Observations of reduced testosterone levels and germ cell alterations in testes of fetal rats exposed to DEHP during gestation [3,12] have led to the hypothesis that perinatal DEHP treatment may induce apoptosis of fetal germ cells. Due to reports of low testosterone levels also postnatally, apoptosis levels were assessed in fetal, prepubertal

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and adult rat testes. Apoptosis measurements following perinatal phthalate exposure have not previously been described in the literature.

The plasticizer di(2-ethylhexyl) adipate (DEHA) shares similarities in chemical structure and metabolism with DEHP, and a comparable profile of effects on the male reproductive system may be suspected. DEHA does not appear to induce adverse effects on the reproductive system but rather developmental toxicity when administered to pregnant dams [13]. As the developmental toxicity of DEHA as well as DEHP may be ascribed to their common metabolite 2-ethylhexanol (2-EH) [14,15], it may be hypothesized that 2-EH is also responsible for some aspects of reproductive toxicity. Thus, DEHA may hypothetically augment DEHP-induced changes in male reproductive endpoints when the two compounds are administered in combination, even though DEHA does not produce these effects on its own.

The aim of the current study was to characterize DEHP-induced early testicular effects by evaluation of testicular histopathology and immunohistochemical studies at gestation day (GD) 21, whereas the study by Jarfelt et al. [2] focuses on the adult effects of DEHP and DEHA. A second aim was to examine apoptosis levels at GD 21, postnatal day (PND) 22, 26 and 190. The application of methods in which the apoptosis-specific Caspase-3 enzyme was detected in situ (immunohistochemistry) and in whole testis homogenate (activity measurement) were compared to methods detecting apoptotic DNA strand breaks in situ (TUNEL staining) and on whole tissue homogenate (DNA laddering). Furthermore, we aimed to reveal if the testicular effects of DEHP were modulated by administration of DEHA in combination with DEHP.

2. Methods

2.1. Test compounds

DEHP, di(2-ethylhexyl) phthalate, CAS No. 117-81-7, purity 99%, was from Sigma-Aldrich 20,115-4. DEHA, di(2-ethylhexyl) adipate, CAS No. 103-23-1, purity 99%, was from Sigma-Aldrich 29,118-8. The vehicle was sterile peanut oil obtained from the Royal Veterinary Agriculture Pharmacy, Copenhagen, Denmark and from Sigma.

2.2. Animals and dosing

2.2.1. Experiment 1

Fourty-eight time-mated Wistar rats (HanTac:WH, Taconic M&B, Denmark, bodyweight approximately 200 g) were supplied at day 3 of pregnancy. The day following mating was designated GD 1, and PND 1 was the day following birth. The dams were randomized into three groups of 16 with similar body weight distributions and housed in pairs until GD 21 under standard conditions: Semi-transparent plastic cages with pinewood bedding were situated in an animal room

with controlled environmental conditions (12 h light–dark cycles with light starting at 6 p.m., light intensity 500 lx, temperature $21 \pm 2^\circ\text{C}$, humidity $50 \pm 5\%$, ventilation eight air changes per h). Food (Altromin Standard diet 1324) and acidified tap water were provided ad libitum. The animals were dosed with either vehicle, 750 mg DEHP/kg bw/day, or 750 mg DEHP/kg bw/day in combination with 400 mg DEHA/kg bw/day from GD 7 to GD 21. Animals were inspected for general toxicity twice daily. On GD 21, eight dams per group were anesthetized in CO_2/O_2 and decapitated, and fetuses were removed and decapitated. The remaining eight dams per group received similar doses as during pregnancy until PND 17, and male offspring was sacrificed at PND 26. Testes were removed from all fetuses and 26-day-old rats and treated as described below.

2.2.2. Experiment 2

Eighty time-mated Wistar rats were randomized into four groups of 20 receiving either 0, 300 or 750 mg DEHP/kg bw/day, or 750 mg DEHP/kg bw/day in combination with 400 mg DEHA/kg bw/day within the same period as described for the first study (GD 7 to PND 17). Housing conditions were as described for the previous experiment. Three males per litter were sacrificed at PND 22, and further one or two males per litter at PND 190. Testes were removed and treated as described below.

The doses in the combination group were selected as the highest doses where no maternal toxicity and a low level of developmental toxicity were seen in previous experiments. In our previous dose-response study of DEHA, 400 mg DEHA/kg bw/day caused a small increase in postnatal death of offspring and no maternal toxicity [13]. The dose of 750 mg DEHP/kg bw/day was similar to the dose used by Parks et al. (2000) [1] and Gray et al. (2000) [3], while the dose of 300 mg DEHP/kg bw/day was selected in order to obtain submaximal effects compared to 750 mg DEHP/kg bw/day.

2.3. Histopathology, TUNEL assay and immunohistochemistry

Not all animals were used for histopathology, as some randomly selected testes were collected for hormone analyses [12] and gene expression studies. Fourteen to 19 testes per group at GD 21 (i.e. 2–4 testes per litter), and 10 testes per group at PND 22 and 26 (i.e. 1–2 testes per litter), were fixed in neutral buffered formalin and embedded in paraffin. 10 testes per group at PND 22 and 26, and 16 testes per group at PND 190 (i.e. 1–2 testes per litter) were fixed in Bouin's fixative. These testes were randomly selected from each of the 5–8 litters per group at GD 21 and PND 26, and the 8–13 litters per group at PND 22 and 190. One section per testis was stained with hematoxylin and eosin and evaluated for histopathological effects in a blinded manner (GD 21 and PND 26). Chord diameters were investigated in fetal testes stained for smooth muscle actin (SMA) by measuring the

diameter of tubular cross sections perpendicular to the tubular length direction. Chord diameters more than 10% larger than the maximal chord diameter in fetal control testes were defined as enlarged chords. In each section, all chords were measured. As some testes (from all groups) were flattened due to technical difficulties, chord diameter was only measured in testes of optimal quality (9–13 testes from 5 to 6 litters per group).

In order to study the apoptosis/proliferation balance at different ages, TUNEL staining of apoptotic cells was performed in addition to immunostaining for the apoptosis-specific enzyme Caspase-3 and the proliferation markers proliferating cell nuclear antigen (PCNA) and Histone H3. Additionally, structural changes were studied using cell type specific markers anti-Müllerian hormone (AMH, Sertoli cell specific) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD, Leydig cell specific) and the structural proteins vimentin and smooth muscle actin.

TUNEL staining was performed on 4 μ m sections fixed on SuperFrost+ slides (Menzel-Gläser, Germany) at 37 °C overnight and then deparaffinized with petroleum and ethanol to water before the staining procedure. The TUNEL assay was performed with ApopTag-peroxidase kit (Cat. No. S7100, Intergen Co, NY) following the supplier's guidelines. Sections from fetal testes were pretreated with 5 μ g/ml Proteinase K in PBS for 5 min, while sections from 22-, 26-, and 190-day-old rat testes were microwave-pretreated in citrate buffer pH 6 for 2 \times 5 min at 99 °C.

Except for pretreatment procedures and secondary antibodies, immunohistochemistry was performed using comparable protocols for all antibodies on one section per testis. Following pretreatment, sections were blocked for endogenous peroxidase activity in 3% H₂O₂ in PBS, and blocked in swine serum with bovine serum albumin. Sections were incubated with primary antibody over night at 4 °C, and were then incubated with secondary antibody for 30 min, stained in 3-amino-9-ethylcarbazole (AEC, Labvision, CA) and counterstained in Meyer's hematoxylin.

Immunohistochemical staining was performed using primary antibodies against cleaved, active Caspase-3 (Cell Signaling Technology, MA, #9661), PCNA (clone PC10, DAKO), phospho-histone H3 (Upstate Biotech), vimentin (clone V9, DAKO), AMH (also called Müllerian inhibiting substance, MIS, DAKO), SMA (clone 1A4, DAKO) and 3 β -HSD (a kind gift from Dr. I. Mason, Edinburgh, UK). Sections for PCNA staining were not pretreated, while sections for Caspase-3 or Vimentin staining were microwave pretreated for 2 \times 5 min in citrate buffer pH 6 at 99 °C, and sections for AMH, SMA and 3 β -HSD staining were pretreated for 5 min in citrate buffer pH 6 at 99 °C. Sections for Histone H3 staining were pressure cooked for 10 min in citrate buffer pH 6. As secondary antibodies were used anti-rabbit EnVision+ (DAKO) for Caspase-3 and 3 β -HSD staining, anti-mouse EnVision+ (DAKO) for PCNA, Vimentin and SMA staining, and rabbit anti-goat antibody (DAKO) enhanced with anti-rabbit EnVision+ for AMH

staining. Biotinylated swine-anti rabbit secondary antibody was used for Histone H3 staining and followed by incubation with AB complex. Double staining for SMA and 3 β -HSD was performed by simultaneous incubation with the two primary antibodies followed by two consecutive staining procedures: first incubation with anti-mouse EnVision+ and staining for SMA with AEC, then blocking in H₂O₂, incubation with anti-rabbit EnVision+ and staining with Vector SG peroxidase staining kit (Vector Laboratories, CA).

Apoptotic TUNEL positive or Caspase-3 positive cells, and proliferating Histone H3 positive cells were quantified in one section per fetal testis. Interstitial and intra-tubular positively stained cells were counted separately throughout the entire section. At PND 22, 26, and 190, the number of TUNEL positive cells was counted in 100–200 tubules in one section per testis from 10 to 20 males per group.

2.4. Apoptosis assessment

Caspase-3 activity was measured on one testis from 10 males per group (from 5 to 10 litters per group) at GD 21, PND 22 and PND 26 as described by Gorman et al. [16] and determined as nmol of substrate cleaved per gram tissue per hour. Double determinations were made for each sample in wells with and without inhibitor.

DNA laddering was performed as described by Staley et al. [17] on one testis from one to three fetuses, 22- or 26-day-old males per litter (10–19 males from 5 to 10 litters per group). DNA was extracted using QIAGEN DNeasy tissue kit and PCR was performed using ApoAlert LM-PCR Ladder Assay Kit (Cat.No. 905-1, Clontec, CA), and Clontech Advantage cDNA Polymerase Mix containing KlenTaq DNA Polymerase, and TaqStart Antibody. Positive controls contained calf thymus DNA, and negative controls did not contain DNA. Results on DNA-laddering are given in arbitrary units, which reflect the relative fluorescence of DNA ladders on gels, and thus are a measure of relative apoptosis level.

2.5. Statistical analysis of apoptosis data

Statistical analyses were performed on apoptosis data. Non-processed and logarithmically transformed data were examined for normal distribution and homogeneity of variance. Following appropriate transformation, data were analyzed by ANOVA using single animal data nested within litter, which was the statistical unit. Litter was included in the analysis of variance as an independent, random factor (SAS Analyst: mixed procedure, SAS Version 8, SAS Institute Inc, Cary, NC, USA). Dunnett's tests were performed to determine differences between treated and control group means. In cases where normal distribution and homogeneity of variance was not obtained, data were additionally tested with the non-parametric Kruskal–Wallis test (correction for litter

effect not possible). Asterisks indicate a statistically significant difference compared to controls (*) $p < 0.05$.

3. Results

3.1. Testicular histopathology, GD 21

Vacuolization of Sertoli cells, germ cell shedding and reduction of Leydig cell cytoplasm were observed in almost all DEHP-exposed and DEHP + DEHA-exposed fetuses, when evaluating one HE stained section per testis. Multinuclear gonocytes and large Leydig cell clusters (apparently hyperplasia of Leydig cells) were observed in approximately half the exposed animals at GD 21 (Table 1, Fig. 1A–C). The Leydig cell clusters contained small cells with irregular nuclei and reduced cytoplasm compared to Leydig cells in control testes. All fetuses exposed to DEHP or DEHP + DEHA had one or more seminiferous chords with increased diameters compared to controls. The diameters of these enlarged seminiferous chords were increased by 19–103% compared to the maximal chord diameter measured in controls (63 μm). This equals a 67–184% increase in chord diameter compared to average chord diameter in controls (45 μm). Inside these enlarged seminiferous chords, germ cells had a reduced cytoplasm and the number of germ cells per chord cross-section appeared to be increased (Fig. 1C).

PCNA staining was present in most gonocytes, Sertoli cells and Leydig cells of fetal control testes, while areas of Leydig cell hyperplasia in DEHP exposed testes were negative for PCNA (Fig. 2A and B). The proliferation marker Histone H3 was mainly present in peritubular cells, interstitial cells and Sertoli cells, and was less abundant than PCNA (not shown). In DEHP exposed animals, Leydig cell clusters and seminiferous chords with an apparent increase in germ cell number did not have increased numbers of Histone H3 positive cells (Table 1).

Staining for 3 β -HSD strengthened the observation of focal Leydig cell clustering instead of the localization as single cells distributed throughout the interstitium in controls (Fig. 2C and D). The intensity of staining did not generally appear to be affected by treatment.

Staining for the cytoplasmic Sertoli cell marker AMH indicated the presence of Sertoli cells within Leydig cell clusters in DEHP and DEHP + DEHA exposed testes (Fig. 2F). In control testes, AMH staining was located throughout seminiferous chords (Fig. 2E), but in chords with shedding of germ cells AMH was found mainly peripherally (Fig. 2F bottom right corner).

SMA staining was strong in peritubular myoid cells and perivascular cells, and there was generally no difference in SMA immunostaining intensity between treatment groups, although a few exposed testes had weakly stained tubules (Fig. 2G and H).

3.2. Testicular histopathology, PND 26

At PND 26, no germ cell shedding, multinucleated germ cells, or vacuolization of Sertoli cells were present, and only small areas of Leydig cell hyperplasia (smaller than the size of a cross-section of a tubule) were observed in a few animals. Seminiferous tubules lacking spermatocytes were observed at PND 26 in rats exposed to DEHP or DEHP + DEHA (Table 1). Malformed tubules were present in 10–15% of testis sections from the DEHP- or DEHP + DEHA exposed rats at day 26, when examining one section per testis (Table 1). Characteristic for these malformed structures were the absence of normal epithelial structure, and anastomoses between tubular structures within the region.

Localization of vimentin staining in Sertoli cells was evaluated in one section per testis, and no differences between groups were observed in fetal or prepubertal rat testes (data not shown). Whereas vimentin filaments in juvenile and adult

Table 1

Histopathological effects in testes of 21-day-old fetal rats and 26-day-old rats exposed from GD 7 to PND 17 to either 750 mg DEHP/kg bw/day, or 750 mg DEHP/kg bw/day in combination with 400 mg DEHA/kg bw/day

	Control	DEHP 750	DEHP 750 + DEHA 400
Histopathological evaluation at GD 21			
Number of animals (number of litters)	17 (7)	14 (8)	19 (5)
Vacuolization of Sertoli cells (%)	6 ^a (14) ^b	100 (100)	100 (100)
Shedding of gonocytes (%)	0 (0)	100 (100)	100 (100)
Multinucleated gonocytes (%)	0 (0)	64 (75)	75 (80)
Reduced cytoplasm in interstitial cells (%)	0 (0)	100 (100)	89 (100)
Presence of chords with enlarged diameter ^c (%)	0 (100)	100(100)	100(100)
Leydig cell clusters/hyperplasia (%)	12 (29)	50 (63)	42 (60)
Histone H3 positive cells per section (mean \pm S.D.) ^d	80 \pm 25	90 \pm 17	71 \pm 28
Histopathological evaluation at PND 26			
Number of animals (number of litters)	20 (7)	20 (6)	20 (7)
Presence of malformed tubules (%)	0 ^a (0) ^b	10 (17)	15 (29)
Presence of tubules lacking spermatocytes (%)	10 (29)	50 (100)	25 (57)

^a Percentage of affected males of the total number of males when evaluating one section per testis.

^b In parentheses are given percentage of affected litters of total number of litters when evaluating one section per testis.

^c Based on 9–13 animals (from 5 to 6 litters) per group.

^d Based on 6–12 animals per group.

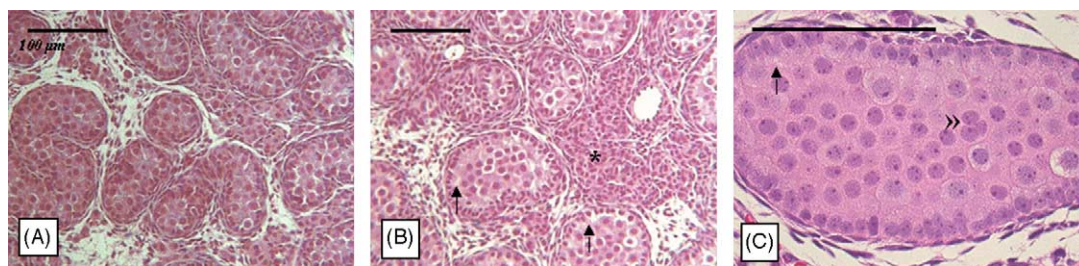


Fig. 1. Testicular histopathology in 21-day-old male rat fetuses exposed to vehicle (A), 750 mg DEHP/kg bw/day (B), or 750 mg DEHP/kg bw/day in combination with 400 mg DEHA/kg bw/day (C) from GD 7 to GD 21. The number of germ cells and the chord diameter appeared increased in seminiferous chords of DEHP- and DEHP + DEHA exposed animals. (↑) Vacuolization in Sertoli cell cytoplasm, (») multinucleated gonocyte, (*) interstitial cell hyperplasia. Scalebar: 100 µm.

rats extended from the Sertoli cell nucleus towards the tubule lumen, vimentin in fetal Sertoli cells was mainly located basally to the nucleus. In prepubertal testes, PCNA staining did not appear to differ between groups.

3.3. Apoptosis

Statistically significant increases in apoptosis level as measured with DNA-laddering were observed at GD 21 in the DEHP and DEHP + DEHA groups compared to controls (Fig. 4). No statistically significant differences between groups in the intensity of bands on DNA ladders were observed at PND 22. At PND 26, DNA-laddering levels in the DEHP + DEHA combination group were statistically significantly higher than in the control group at PND 26, while the group receiving 750 mg DEHP/kg bw/day was not statistically significantly different from the control group or the DEHP + DEHA combination group (Fig. 4).

The number of TUNEL and Caspase-3 positive gonocytes and interstitial cells appeared higher in DEHP + DEHA treated fetuses compared to controls, though the number of positive cells per section was very low, on average five positive cells per section (Fig. 4). The number of Caspase-3 positive cells matched the number of TUNEL positive cells at GD 21, whereas very few cells were Caspase-3 positive at PND 22 compared to TUNEL (data not shown). The numbers of TUNEL positive cells per tubule were not significantly different between groups at PND 22, 26 or PND 190. However, at PND 22 as well as 190, a few animals in the treated groups had very high numbers of TUNEL positive cells, presumably spermatocytes (Fig. 3). At PND 22, high apoptosis levels were seen in testes where many tubules were lacking spermatocytes (Fig. 3B), and where large areas of dysgenic tubules were present in the same section. In adults, increased numbers of TUNEL positive cells were seen mainly in atrophic testes. Specifically, the apoptotic cells were found in tubules with ongoing spermatogenesis, whereas end-stage tubules lacking spermatocytes had few TUNEL positive cells (Fig. 3D).

Caspase-3 activity was not detectable at GD 21 in testes of controls or treated animals. No statistically significant differences between groups in Caspase-3 activity at PND 22 or 26 were observed (not shown).

4. Discussion

The current studies aim to relate the early testicular effects of perinatal exposure of male rats to DEHP and DEHA to the adult effects described by Jarfelt et al. [2]. The progress of testicular lesions was studied by an evaluation of the apoptosis-proliferation balance and immunohistochemical characterization of testicular lesions in fetal, prepubertal and adult rat testes.

Confirming findings in studies on DBP-exposed fetuses [4–6], histopathological effects were observed in most testes in late gestation. Testes of DEHP and DEHP + DEHA exposed fetal rats had vacuolization of Sertoli cells, shedding of gonocytes, presence of multinucleated gonocytes, an apparent increase in tubule diameter with an increased number of gonocytes with small cytoplasm, as well as an apparent increase in the number of Leydig cells with a reduced amount of cytoplasm (Fig. 1 and Table 1).

4.1. Early origins of adult testicular effects

Staining for the Sertoli cell product AMH in testes of DEHP-exposed fetuses revealed the presence of Sertoli cells outside seminiferous chords in areas of Leydig cell hyperplasia (Fig. 2F). As described by Jarfelt et al. [2], dysgenic areas characterized as malformed tubules within focal areas of interstitial cell hyperplasia were observed at PND 22 and 190 in DEHP-exposed rats. In addition to Sertoli cells and a reduced number of germ cells, Leydig cells positively stained for 3β-HSD were found inside these tubules.

It is likely that the development of dysgenic tubules is related to the morphological changes in the interstitium observed already during gestation. Fisher et al. [6] have shown the presence of incomplete chords lined with smooth muscle actin within dysgenic areas as early as PND 4 in rats exposed to DBP from GD 13.5 to 21.5. They found gonocytes, Sertoli cells and Leydig cells within these malformed chords, as determined by immunohistochemical staining for proteins specific for these cell types. In this study, no dysgenic chords were observed at GD 21 with SMA staining, and no 3β-HSD positive Leydig cells were found inside seminiferous chords. This difference to the findings by Fisher et al. [6] indicates

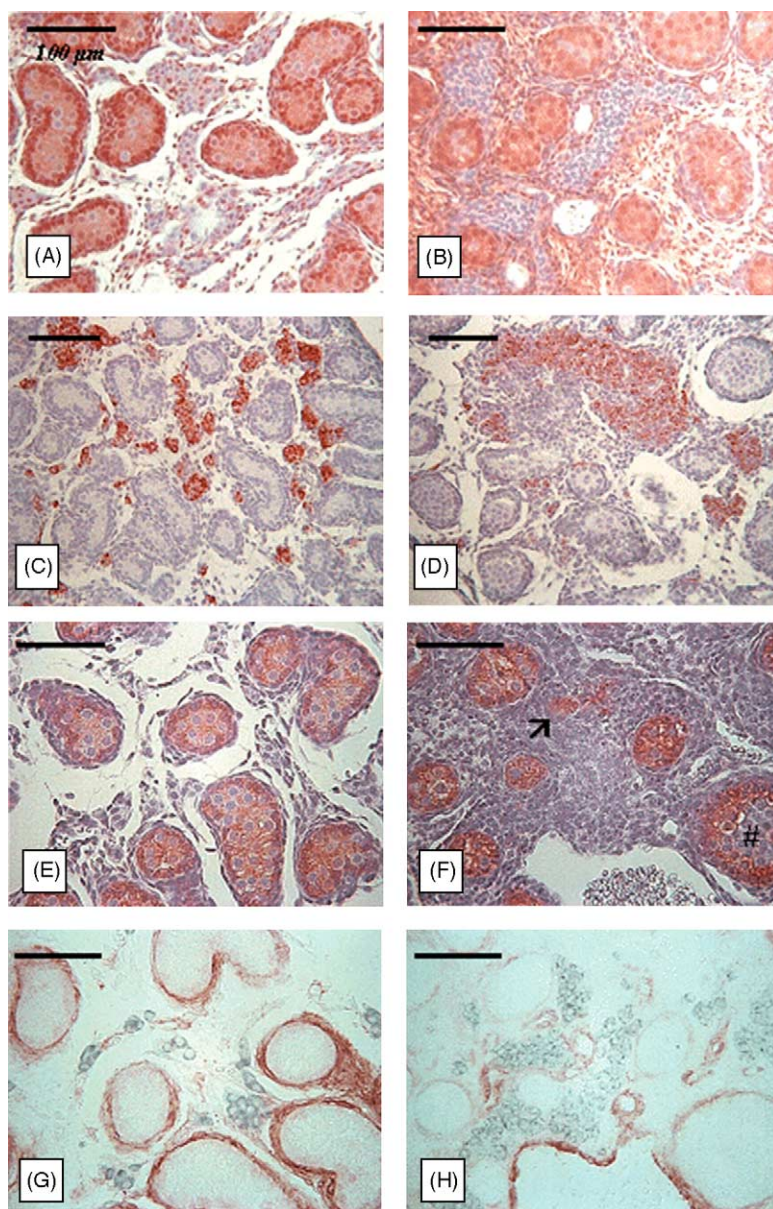


Fig. 2. Immunohistochemical staining of testes of 21-day-old male rat fetuses exposed to 750 mg DEHP/kg bw/day + 400 mg DEHA/kg bw/day from GD 7 to GD 21 (B, D, F, H). (A, C, E, and G) are control rat testes. Interstitial cells in hyperplastic areas are negative for PCNA staining and surrounded by positively stained mesenchymal cells (B). Staining for localization of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) shows positively stained Leydig cell clusters surrounded by mesenchymal cells negative for 3 β -HSD (D). Staining for the Sertoli cell specific marker Anti-Müllerian Hormone indicated the presence of Sertoli cells (\rightarrow) within Leydig cell clusters (F). (#) Indicates tubules with shedding of germ cells and a peripheral localization of AMH staining (F). Double staining for 3 β -HSD and α -smooth muscle actin (SMA, specific for peritubular and perivascular cells) shows weak SMA staining in peritubular cells of some tubules neighboring Leydig cell clusters (H). D + H are serial sections. Scalebar: 100 μ m.

that the dysgenic chords develop between GD 21 (our observations) and PND 4 (their observations). The development of dysgenic tubules may indicate malfunction of the androgen-dependent peritubular cells, though not much is known about the role of androgens and peritubular cells in tubular formation. Fisher et al. described a reduced intensity of SMA staining at GD 19 but not at GD 15, 17 or PND 4 in DBP exposed rats, indicating defects of peritubular myoid cells [6]. Such reduction of staining intensity was generally not confirmed at GD 21 in the present study with DEHP, although some ar-

reas of the testes had weaker staining than normal (Fig. 2H). These areas were neighboring Leydig cell clusters with displaced Sertoli cells, indicating that these areas may develop into malformed chords by PND 4.

Previous studies have shown a reduction of testosterone production by DEHP-exposed rats [3,12]. This leads to an increase in LH [12] and may cause the assumed Leydig cell proliferation as an attempt to compensate for the reduced testosterone levels. In the current study, no quantification of Leydig cells was performed, but Leydig cell number appeared

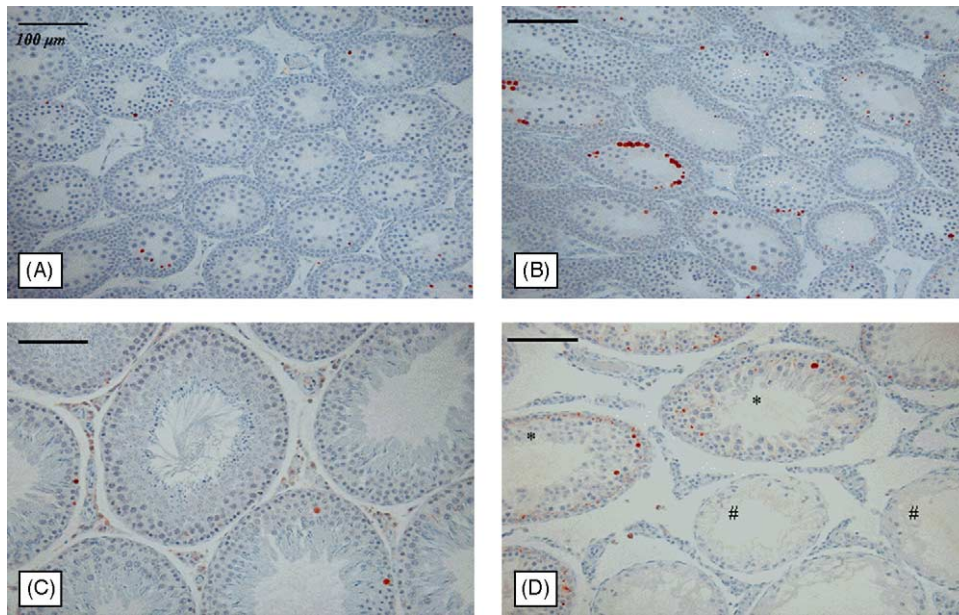


Fig. 3. TUNEL staining of apoptotic cells in testes of 22-day old (A + B) and 190-day-old (C + D) rats exposed to 750 mg DEHP/kg bw/day from GD 7 to PND 17. The number of apoptotic germ cells (red staining) is increased in some but not all 22-day old rat testes (B). In atrophic adult testes (D), apoptosis levels are high in tubules with some spermatogenesis (*), whereas end-stage tubules (#) have few apoptotic cells. Scalebar: 100 μ m.

high not only in DEHP-exposed males, but also in several controls. However, the normal testes had Leydig cells with an “active” appearance with a large, vacuolated cytoplasm and a large spherical nucleus, and were found in small groups or as single cells, while Leydig cells in DEHP-exposed testes appeared “inactive” as they were small and found in large clusters (Fig. 1A and B). If the assumed Leydig cell hyper-

plasia in DEHP-exposed males is real, the lack of PCNA staining (Fig. 2B) indicates that any increase in Leydig cell proliferation rate has apparently stopped before GD 21. Cells surrounding the PCNA-negative Leydig cell clusters had intense PCNA-staining, and as these cells were 3 β -HSD negative they may be undifferentiated mesenchymal cells (Fig. 2B and D). At this age, the apparent hyperplasia may thus take

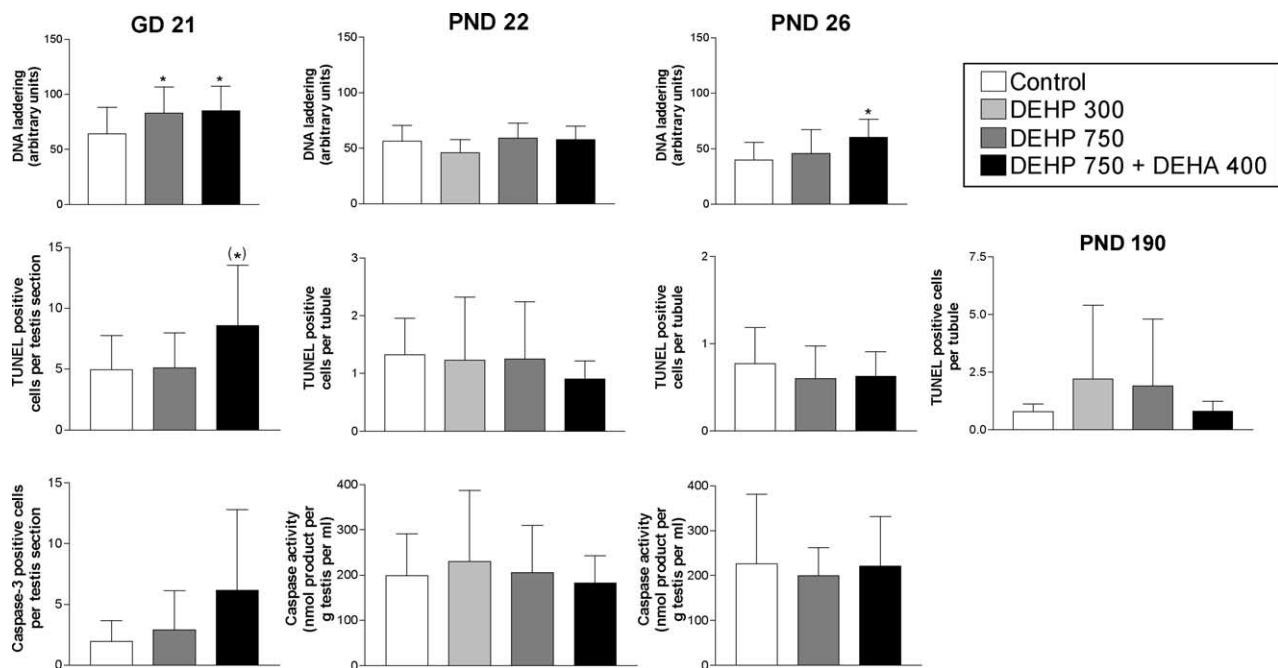


Fig. 4. Apoptosis levels in testes of rats exposed from GD 7 to PND 17 to either vehicle, 300 mg DEHP/kg bw/day (DEHP 300), 750 mg DEHP/kg bw/day (DEHP 750), 750 mg DEHP/kg bw/day + 400 mg DEHP/kg bw/day (DEHP 750 + DEHA 400). Values are means \pm SD. (*) indicates statistically significant differences from controls, $p < 0.05$.

place in the undifferentiated cells, as suggested by Mylcreest et al. [5].

The testosterone reduction may affect neighboring cells in the testes as well as other organs. An early defect in Sertoli cell maturation may have consequences for later spermatogenesis. Alternatively, late testicular atrophy may be indirectly caused by Leydig cell dysfunction, as low testosterone levels may cause epididymal dysgenesis and secondary effects on the testes, as suggested by Barlow and Foster [4]. Focal dysgenesis in testes may rather originate from fetal or early postnatal development, and is possibly related to Sertoli cell dysfunction, as immature Sertoli cells were described by Fisher et al. [6] in abnormal seminiferous tubules of adult DBP exposed rats.

4.2. Germ cell apoptosis and proliferation from gestation to adulthood

Despite the observed loss of contact between germ cells and Sertoli cells in fetal testes (Fig. 1) and the reduced fetal testosterone levels [12], apoptosis levels did not appear markedly increased in the examined fetal testes. This is in contrast to the germ cell apoptosis seen after acute phthalate exposure of adult or prepubertal rats [8,9]. A tendency to increased numbers of TUNEL or Caspase-3 stained cells within the seminiferous chords of DEHP and DEHP + DEHA treated fetuses was confirmed by increased DNA-laddering. However, very few of the shedded gonocytes in fetal testes were Caspase-3 positive or TUNEL positive (Fig. 4). In fact, the number of gonocytes appeared to be increased within each tubular cross-section (Fig. 1C). As the number of cells positive for the proliferation marker Histone H3 did not differ between groups when assessed in one section per testis, any possible increase in gonocyte number may be due to proliferation at an earlier age. As apoptosis levels were found to be low, the shedded germ cells probably die at a later age, e.g. concomitantly with the multinucleated gonocytes, which disappear before PND 10 in phthalate-exposed testes [1,4,18]. Despite the differences found with DNA-laddering, apoptosis does not appear to be an important feature of neither DEHP nor DEHA exposure at GD 21 at the applied doses. Opposite findings in prepubertally exposed males [9], the observed DEHP-induced disruption of contact between fetal germ cells and Sertoli cells was apparently not related to collapse of the structural vimentin filaments, as no differences in vimentin structure between controls and DEHP-exposed fetal testes were observed.

At puberty, testicular apoptosis levels were normal in most DEHP-exposed rats. The few testes with increased apoptosis level at PND 22 also contained dysgenic tubules and tubules lacking spermatocytes. The changes seen in these 22-day-old testes may be related to adult testicular dysgenesis, whereas animals with apparently normal testes in prepuberty likely develop normally into adulthood.

Poor Sertoli cell function may cause germ cell apoptosis [19], and as phthalates are known to disrupt Sertoli cell func-

tion [20–22], apoptosis in the degenerating testes and in dysgenic tubules may be due to Sertoli cell dysfunction. In a study on acute MEHP treatment of 28-day-old rats, condensation of the structural vimentin filaments in Sertoli cells was seen 3 h after treatment followed by an increase in apoptosis levels 6 h after dosing [9]. In the current study, there was a general lack of effect on vimentin structure at PND 22. Vimentin filaments had a disorganized distribution within dysgenic tubules, but appeared normal in other tubules at this age. The apparently normal Sertoli cell structure in prepubertal males in the current study may reflect recovery after the end of dosing at PND 17, or a gradual adjustment to the effects of chronic perinatal phthalate exposure. If the effect of prepubertal (in this case lactational) exposure to DEHP is indeed degenerative as shown in studies on 28-day-old MEHP-exposed rats [9,23], any possible increase in cell death may be reversible due to the rapid turnover of cells in the testicular epithelium and therefore absent in most animals at the examined ages PND 22 and 26, i.e. 4–8 days after the end of lactational exposure.

In adults, apoptosis levels were increased in a few testes with atrophic seminiferous epithelium, whereas no statistically significant difference was seen between group means. The ongoing apoptosis in adult testes with atrophic seminiferous epithelium may indicate that spermatogenesis does occur to some degree, and that new spermatocytes keep appearing only to undergo apoptosis. Conversely, the most degenerated testes with mainly Sertoli cell-only tubules had a relatively low number of apoptotic cells.

A tendency to an apoptosis-inducing effect of DEHA was seen with DNA-laddering in prepubertal rats at PND 26, as apoptosis levels were higher than controls in the combination group, but not in the DEHP group. This may be a random finding, as this is not seen at PND 22, and as TUNEL data on the contrary pointed towards lower apoptosis levels in the exposed groups. Unfortunately, these studies lack a group receiving DEHA alone for comparison with the control and the combination groups. However, our previous studies have shown no testicular histopathological changes or malformations of reproductive organs in male rats gestationally and lactationally exposed to DEHA (up to 1200 mg/kg bw/day) [13].

In conclusion, apoptosis did not appear to be an important element of the testicular effects of perinatal exposure to DEHP or to DEHP in combination with DEHA in fetal testes, but in prepubertal and adult testes, apoptosis was involved in the degeneration of seminiferous tubules. Histopathological effects were observed in most testes in late gestation, although only a few prepubertal and adult testes were severely affected. DEHP has similar effects to those described for DBP by others [4,6]: At GD 21, Leydig cells were found in large clusters containing displaced Sertoli cells, and seminiferous chords were enlarged and appeared to have an increased number of centrally located gonocytes, although gonocyte proliferation was not increased at this age. Focal dysgenesis in testes likely originates from fetal or early postnatal development, whereas general testicular atrophy may be due to either dysfunctional

Sertoli or Leydig cells or be secondary to effects of androgen deficiency in other reproductive organs. No significant differences between DEHP and DEHP + DEHA exposed rats were detected.

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Antiandrogenic effects in male rats perinatally exposed to a mixture of di(2-ethylhexyl) phthalate and di(2-ethylhexyl) adipate

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Abstract

Di(2-ethylhexyl) phthalate (DEHP) is a well-known testicular toxicant inducing adverse effects in androgen responsive tissues. Therefore, di(2-ethylhexyl) adipate (DEHA) is currently being evaluated as a potential substitute for DEHP. Similarities in structure and metabolism of DEHP and DEHA have led to the hypothesis that DEHA can modulate the effects of DEHP. Wistar rats were gavaged with either vehicle, DEHP (300 or 750 mg/kg bw/day) or DEHP (750 mg/kg bw/day) in combination with DEHA (400 mg/kg bw/day) from gestation day (GD) 7 to postnatal day (PND) 17.

Decreased anogenital distance (AGD) and retention of nipples in male offspring were found in all three exposed groups. Dosed males exhibited decreased weights of ventral prostate and *m. levator ani/bulbocavernosus*. Histopathological investigations revealed alterations in testis morphology in both juvenile and adult animals. The litter size was decreased and postnatal mortality was increased in the combination group only, which is likely a combined effect of DEHP and DEHA. However, no combination effect was seen with respect to antiandrogenic effects, as males receiving DEHP in combination with DEHA did not exhibit more pronounced effects in the reproductive system than males receiving DEHP alone.

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1. Introduction

The increase in developmental and functional disorders of the male reproductive tract in both humans and wildlife have led to the hypothesis that perinatal exposure to environmental chemicals may be harmful to normal development of the male reproductive tract by disrupting the endocrine system [1,2]. In 1992, Carlsen and co-workers suggested a worldwide decline in human sperm quality and it was proposed that environmental chemicals might be partly responsible [3,4]. Large geographical differences in semen quality have

recently been reported both in the US and in northern Europe [5,6].

Phthalates are plasticizers that induce flexibility to polyvinyl products in several consumer products [7]. Di(2-ethylhexyl) phthalate (DEHP) is one of the most commonly used plasticizer and humans are exposed via oral, inhalation and intravenous routes [8–10]. DEHP and DBP are well known testicular toxicants in rodents inducing adverse effects in androgen responsive tissues [11–13], and low sperm counts [12,14,15]. A study by Duty and co-workers demonstrated an association between phthalate levels and impaired sperm quality in humans [16]. The development of the male reproductive tract is androgen dependent and therefore vulnerable to antiandrogens. It is suggested that the effects of

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phthalates are caused by an antiandrogenic mechanism not involving the androgen receptor [17,18], and recent studies report that DINP, DBP and DEHP decrease testosterone synthesis in foetal testes [18–20].

Because of the antiandrogenic effects of DEHP, di(2-ethylhexyl) adipate (DEHA) is being evaluated as a potential substitute for DEHP. We have previously studied the reproductive effects of DEHA [21]. Results showed that DEHA induced mild developmental toxicity at 400 mg/kg bw/day, moderate developmental toxicity from 800 mg/kg bw/day, but no antiandrogenic effect at doses up to 1200 mg/kg bw/day. In spite of these results, similarities in structure and metabolism of DEHP and DEHA have led to the hypothesis that similarities in action may exist. Therefore, the aim of the present study was to examine whether antiandrogenic effects of perinatal exposure to DEHP could be modulated by DEHA. The doses in the combination group were selected as the highest doses where no maternal toxicity and a low level of developmental toxicity were seen in previous experiments. In our previous dose–response study of DEHA, 400 mg DEHA/kg bw/day caused a small increase in postnatal death of offspring and no maternal toxicity [21]. The dose of 750 mg DEHP/kg bw/day was similar to the dose used by Parks et al. and Gray et al. in comparable study designs [12,18]. The dose of 300 mg DEHP/kg bw/day was selected in order to obtain submaximal effects compared to 750 mg DEHP/kg bw/day. As few studies on semen quality following perinatal DEHP exposure of rats have yet been reported, we examined semen production as well as velocity and motility parameters. Additionally, we aimed to verify previous findings of irreversible effects of DEHP by evaluating the histopathology of reproductive organs and the typically antiandrogenic effects on anogenital distance and nipple retention.

2. Materials and methods

2.1. Chemicals

DEHP, CAS No. 117-81-7, purity 99% and DEHA, CAS No. 103-23-1, purity 99% was obtained from Sigma–Aldrich, St. Louis, MO. The vehicle was sterile peanut oil obtained from the Royal Veterinary and Agriculture Pharmacy, Copenhagen, Denmark.

2.2. Animals and treatment

Eighty time-mated, young adult Wistar rats with a body weight around 200 g (HanTac:WH, Taconic M&B, Denmark) were supplied at day 3 of pregnancy. Upon arrival, the females were randomly distributed in pairs and housed under standard conditions: semitransparent plastic-cages (15 cm × 27 cm × 43 cm) with pinewood-bedding (Hanflock H9), situated in an animal room with controlled environmental conditions (12 h light–dark cycles with light starting at 6

p.m., light intensity 500 lux, temperature $21 \pm 2^\circ\text{C}$, humidity $50 \pm 5\%$, ventilation 8 air changes per hour). Food (Altromin Standard Diet 1324) and acidified tap water were provided *ad libitum*.

At gestation day (GD) 4, the females were weighed and assigned to four groups, 20 dams per group, with similar weight distributions. The four groups of dams were gavaged daily with vehicle, 300 or 750 mg DEHP/kg bw/day, or with 750 mg DEHP/kg bw/day in combination with 400 mg DEHA/kg bw/day in a dosing volume of 2 ml/kg bw from GD 7 to PND 17. At GD 20, the females were housed individually. They were observed twice a day for signs of toxicity. Body weight was recorded on GD 4 and daily during the dosing period.

From GD 21, animals were inspected twice a day at 8 a.m. and 4 p.m. and time of birth was recorded. After delivery, body weights of dams and individual pups were recorded. Pups were counted, sexed and checked for anomalies. Pups found dead were investigated for macroscopic lesions, when possible. The day following delivery was designated PND 1 for the pups. Litter size was not standardized.

One male and one female from each litter were kept after weaning at PND 21, to investigate sexual maturation, sperm quality and histopathology of testes at adulthood. These pups were selected randomly and housed in pairs of the same sex and exposure status. Three to five pups per litter were sacrificed by decapitation following CO₂-anaesthesia at PND 22 for histopathological investigation of the testes. The rest of the pups and dams that had not given birth were sacrificed as well at PND 22 and examined for macroscopic lesions. The number of uterine implantation sites was counted in the dams.

2.3. Postnatal development

Body weight of the offspring was registered in the pre-weaning period at PND 3 and 13. In order to examine feminization of males, anogenital distance was measured and the presence of nipples was examined. Anogenital distance was measured in the offspring on PND 3. On PND 13, the pups were examined for the presence of either areolas or nipples, described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. No distinction was made between the retention of an areola or a nipple.

Age of sexual maturation was investigated by recording the day of vaginal opening in the females and cleavage of the balano-preputial skinfold in the males. The latter was done by observing when the prepuce, which is fused to the glans penis until the onset of puberty, could be fully retracted.

2.4. Sperm analysis

Sexually mature males (PND 190) were anaesthetised by CO₂/O₂ and decapitated. The epididymides were removed

and the cauda of the right epididymis was used for motility analysis. The cauda of the left epididymis was frozen in liquid nitrogen for later sperm count. Sixteen animals per group were used, one to two males per litter.

2.5. Sperm motility

The epididymis was trimmed of fat and cut at the corpus-cauda region. Cauda was placed in a petri dish containing 3 ml warm (37 °C) Medium 199 Hanks & Hepes (Invitrogen Life Technologies, Denmark), supplemented with 0.5% bovine serum albumin (crystallised and lyophilised; Sigma, MO). Spermatozoa were obtained from the distal cauda where the tubular diameter is widest. Under the dissecting microscope, the cauda was held by forceps and several stabs were made into the tubules. The petri dish was placed in an incubator for 5 min. Cauda was removed and the sperm sample was diluted 10 times (in medium 199, supplemented with 0.5% bovine serum) and re-placed in the incubator for 10 min, to allow dispersion of spermatozoa. Sperm samples were loaded into a 100 µm flat cannula (HTR 1099, Fercom, Denmark) and analysed by computer assisted sperm analysis (CASA; HTM-IVOS version 12 Hamilton Thorne Research, Beverly, MA, US). Minimum 20 fields were recorded at 60 Hz under $\times 4$ dark field illumination, and the images were video recorded for later analysis.

The standard set up was used during analysis and tracking errors were deleted through the edit and playback features. Twelve fields (minimum 200 sperm cells) were analysed for each sperm sample. The samples were blinded to the observer. The parameters evaluated in this study were: percent motile and percent progressive spermatozoa, curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) that describe the vigour of the spermatozoa, and some progressive parameters such as average path velocity (VAP), straight line velocity (VSL) and straightness (STR).

2.6. Sperm count

Cauda of the left epididymis was thawed at room temperature, trimmed of fat, weighed and homogenised in 1 ml Triton X-100. The homogenate was stored at 5 °C and treated with ultrasonic sound for 21/2 min the following day. Distilled water (100 µl) was placed into a stain reaction vial containing DNA-specific fluorescent stain (Supra Vital IDENT Stain Kit, HTR) and vortexed. One hundred microlitres of the sperm homogenate was added, vortexed, followed by incubation for 2 min at room temperature. After subsequent vortexing, 3.5 µl was loaded into a 20 µm deep counting chamber (20 Micron, LEJA, Fercom, Denmark). Before the sample was placed in the HTM-IVOS, it was allowed to settle for 2 min. Samples were analysed using 10 \times UV fluorescent objective and IDENT OPTIONS set A. Ten fields were analysed for each sample and three counts were performed for each suspension. Counts were averaged

and data are presented as number of sperm per gram cauda.

2.7. Organ weight, histopathology and immunohistochemistry

All males underwent a thorough autopsy. The following organs were excised and weighed in PND 22 males and adult males: brain, liver, kidneys, adrenals, testes, epididymides, seminal vesicles, ventral prostate, bulbourethral glands, and levator ani/bulbocavernosus muscles. Caput of the left epididymis, ventral prostate, and seminal vesicles were fixed in formalin, embedded in paraffin and examined by light microscopy after staining with haematoxylin and eosin. In PND 22 males, histopathological investigations were performed in one testis from each of around 20 males per group, representing 10 litters. Ten testes were Bouin's fixed, and 10 testes were formalin fixed. The other reproductive organs were investigated in 10 males representing 10 litters per group. In adult males, reproductive organs including testis were investigated in 14–16 adult males representing 14–16 litters per group. All testes from adults were Bouin's fixed. Sections were blinded to the observer with respect to dose groups.

For immunohistochemistry one section per testis were pre-treated for 5 min in citrate buffer pH 6 at 99 °C, and incubated simultaneously with the two primary antibodies against 3 β -hydroxysteroid dehydrogenase (3 β -HSD, a kind gift from Dr. I. Mason, Edinburgh, UK) and smooth muscle actin (SMA, clone 1A4, DAKO, Denmark) over night at 4 °C. This was followed by two consecutive staining procedures: first incubation with anti-mouse EnVision+ and staining for SMA with AEC, then blocking in H₂O₂, incubation with anti-rabbit EnVision+ and staining with Vector SG peroxidase staining kit (Vector Laboratories, CA).

2.8. Statistical analysis

The litter was generally considered the statistical unit and differences were considered statistically significant when *p*-values were lower than 0.05. Pregnancy data and postnatal development were analyzed using ANOVA, when the data showed normal distribution and homogeneity of variance. When relevant, body weight or litter size was included as a covariate in the analyses and a nested design adjusting for litter effects was used. For post-hoc pairwise comparisons Dunnett's test was used. For data not fulfilling the conditions for ANOVA, non-parametric analysis of variance or Fisher's exact test were performed. The SYSTAT PC-version software package (Systat 1990) was used for these statistical analyses.

One to four males per litter from ten to sixteen litters per group were used to analyse sperm quality at PND 190, and terminal body weight and organ weights in PND 22 and PND 190 males. Non-processed and ln-transformed data were examined for normal distribution and homogeneity of variance. In order to adjust for litter effects, litter was included in the analysis of variance as an independent, random, and nested

factor (proc mixed, SAS version 8, SAS Institute Inc., Cary, NC, USA). Organ weights were analysed using treatment as one main factor and age as another main factor and body weight was used as a covariate. When an overall significant treatment effect was observed, two-tailed comparison was performed using least square means. In cases where normal distribution and homogeneity of variance was not obtained, data were additionally tested with the non-parametric Kruskal–Wallis test. Macroscopic lesions and testis effects were analysed using Fisher's exact test. The combination group was compared to the 750 mg DEHP group in order to investigate the possible modulating effect of DEHA.

3. Results

3.1. Pregnancy data, postnatal growth and development

Pregnancy and litter data are summarized in Table 1. No clinical signs of toxicity were observed in the dams during the dosing period. Maternal weight gain from GD 7 to 21 was decreased in dams treated with DEHP alone or with DEHP in combination with DEHA when compared to the control dams. However, the difference was only statistically significant for the group exposed to the combined dosing. Maternal body weight on PND 1 and during the lactation period was similar among groups.

The number of pups per litter and the birth weight of live pups were decreased in litters exposed to DEHP and DEHA in combination. Birth weight was also significantly decreased in pups exposed to 750 mg DEHP. The postimplantation-perinatal loss (i.e. number of implants minus number of live pups at birth) was statistically significantly increased

in litters exposed to 750 mg DEHP and in litters exposed to DEHP and DEHA in combination. Postnatal mortality seemed higher in all exposed groups, but the difference was only statistically significant in litters exposed to DEHP in combination with DEHA. The number of pups dying postnatally were 1 of 94 (1.1%), 5 of 102 (4.9%), 10 of 139 (7.2%), and 28 of 113 (24.8%) in the control group, 300 mg DEHP, 750 mg DEHP, and the combination group, respectively. There were 3 litters with total loss of pups in the group exposed to DEHP and DEHA in combination. These litters consisted of 2, 2 and 6 pups, i.e. 10 dead pups in total, while the other 18 dead pups were distributed among 9 other litters.

The anogenital distances on PND 3 were significantly decreased in the male pups in all exposed groups compared to the control group (Table 1). The male offspring displayed decreases of 14, 17, and 21% in the 300 mg DEHP, 750 mg DEHP, and 750 mg DEHP plus 400 mg DEHA groups, respectively. There was no significant difference in male AGD between the 750 mg DEHP dose group and the combined dose group. The mean AGD for control female pups was 2.38 mm (51% of AGD in control male pups).

On PND 13, the mean number of areolas/nipples was statistically significantly increased in male offspring from litters in all exposed groups (Table 1). The mean number of areolas/nipples in the litters exposed to DEHP and DEHA in combination was significantly lower than the mean values for the litters exposed to the same dose of DEHP alone. The distribution of individual exposed males in relation to number of nipples is shown in Fig. 1. In the control group, 90.9% of the males had 0 areolas/nipples and the rest had only 1. In male offspring exposed to 750 mg DEHP, only around 10% had 0 or 1 nipple, while around 40% had 6–10

Table 1

Reproductive parameters in rats dosed by gavage with DEHP or DEHP in combination with DEHA from GD 7 to PND 17

	Control	DEHP (300 mg)	DEHP (750 mg)	DEHP (750 mg) + DEHA (400 mg)
Number of litters	12	11	15	15
Maternal weight gain GD 7 to 21 (g)	77.3 ± 15.8	70.1 ± 19.9	65.0 ± 10.8	60.7 ± 17.0*
Maternal weight, PND 1 (g)	233.6 ± 16.0	231.3 ± 18.2	228.9 ± 16.3	228.3 ± 15.4
Pregnancy length (days)	22.7 ± 0.4	22.8 ± 0.4	22.8 ± 0.4	22.9 ± 0.5
Birth weight, (g)				
Males	5.93 ± 0.27	6.03 ± 0.43	5.54 ± 0.55*	5.39 ± 0.69*
Females	5.75 ± 0.36	5.81 ± 0.54	5.30 ± 0.55*	5.11 ± 0.54*
Male pups per litter (%)	56 ± 28	61 ± 14	46 ± 23	57 ± 22
Litter size, at birth	10.4 ± 3.2	9.3 ± 2.9	9.3 ± 3.6	7.5 ± 2.9*
Postnatal death, mean	0.11 ± 0.33	0.45 ± 0.93	0.53 ± 0.99	1.87 ± 1.64**
Postimplantation-perinatal-loss (%) ^a	6.7 ± 12.0	12.9 ± 14.1	15.3 ± 12.0*	20.0 ± 15.9**
AGD in males, PND 3 (mm)	4.63 ± 0.14	3.97 ± 0.57**	3.83 ± 0.36**	3.67 ± 0.29**
Body weight, PND 13 (g)				
Males	24.0 ± 2.4	26.0 ± 2.6	23.5 ± 3.7	24.7 ± 3.9
Females	24.3 ± 3.2	25.8 ± 2.9	22.6 ± 3.6	24.2 ± 3.0
Nipples in males, PND 13 (number)	0.1 ± 0.2	3.9 ± 2.7**	5.2 ± 1.7**	3.9 ± 2.4** ⁺

^a (No. implantations – litter size at birth)/no. implantations.

* $p < 5\%$ compared to control group.

** $p < 1\%$ compared to control group.

⁺ $p < 5\%$ compared to group dosed with 750 mg DEHP/kg bw/day.

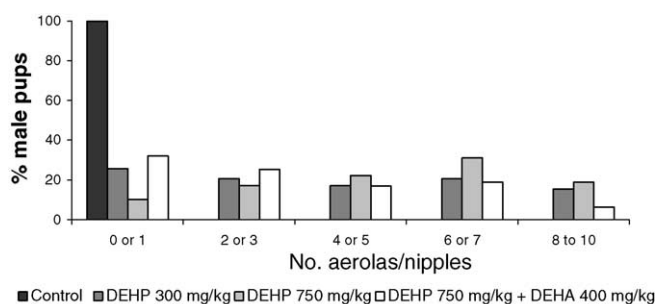


Fig. 1. Areolas/nipples at PND 13 in male rats exposed to either vehicle, DEHP or DEHP in combination with DEHA from GD 7 to PND 17. Values represent the percentage of male offspring in each group with either 0–1, 2–3, 4–5, 6–7 or 8–10 areolas/nipples.

nipples. The distribution in male offspring exposed to 300 mg DEHP or 750 mg DEHP plus 400 mg DEHA seems rather similar with 26–32% having 0–1 nipple and 25–36% having 6–10 nipples.

The age of sexual maturation was similar among groups (data not shown).

3.2. Sperm quality

Fig. 2 shows the number of sperm per gram cauda epididymis. No statistically significant reduction in the number of sperm cells was observed, and no statistically significant reductions were found when investigating any of the sperm motility and velocity parameters compared to the control group. Sperm quality data could not be analysed by a parametric statistical procedure, as the group dosed with 300 mg DEHP had three males with severely reduced sperm number when compared to the control group (Fig. 2). Furthermore, these three males plus two males dosed with 750 mg/kg also had a severely reduced percentage of motile sperm compared to the control group (Fig. 3), as well as considerable reductions in all other sperm motility and velocity parameters tested (data not shown). In order to investigate the effect on the remaining males, the severely affected dosed outliers were excluded from the statistical analyses. No statistically significant effects were observed.

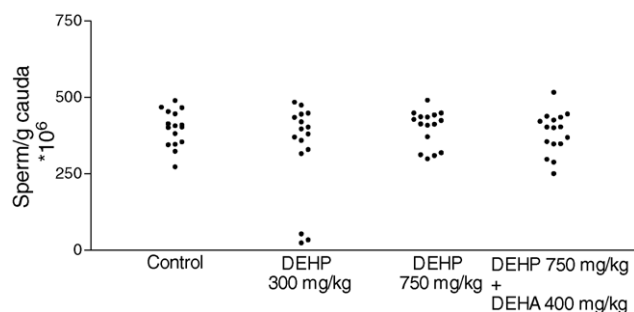


Fig. 2. Sperm number in cauda epididymis in adult male rats exposed to vehicle, DEHP or DEHP in combination with DEHA from GD 7 to PND 17. Values are single animal data, $n = 16$.

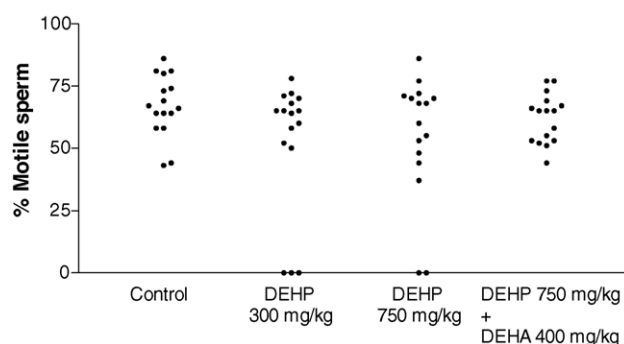


Fig. 3. Sperm motility in adult male rats exposed to vehicle, DEHP or DEHP in combination with DEHA from GD 7 to PND 17. Values are single animal data, $n = 16$.

3.3. Macroscopic findings, terminal body weight, organ weights, immuno- and histopathology

A low incidence of malformations related to the reproductive system was observed in the groups exposed to DEHP or DEHP in combination with DEHA, as listed in Table 2. Microphthalmia was found in one male receiving the high dose of DEHP and two males in the combination group, while hydronephrosis was found in control animals as well as the exposed groups (data not shown). However, no statistically significant differences were found between the exposed groups compared to the control group for any of the observed malformations.

No treatment-related effects on body weight, brain, liver, kidneys or adrenals weights were observed at PND 22 or 190 (Tables 3 and 4). In the statistical analysis of ventral prostate, *m. levator ani/bulbocavernosus* and paired testes weight, an interaction between dose and age was observed. Hence, in PND 22 males, a statistically significant decrease in paired testes weight after receiving 750 mg DEHP was observed, while no decrease in *m. levator ani/bulbocavernosus* or ventral prostate weights were observed (Table 3). This was in contrast to PND 190 males, where statistically significant decreases in weights of ventral prostate and *m. levator ani/bulbocavernosus* were found in all exposed groups (Table 4).

No effect was observed on testis, epididymis and seminal vesicle weights at PND 190. These data could not be analysed by a parametric statistical procedure, as a few of the PND 190 males in the 300 mg DEHP and 750 mg DEHP groups had very small testes and epididymides (Table 4). Additionally, one of the males in the group dosed with 300 mg DEHP had atrophic seminal vesicles. In order to investigate the effect on the remaining males, the severely affected dosed outliers were excluded from the statistical analyses. No statistically significant effects were observed.

Haematoxylin and eosin staining of control testes from PND 22 and PND 190 males are presented in Fig. 4 A and D, respectively. In testes from males dosed with DEHP alone or DEHP in combination with DEHA, normal testis tissue with small foci of malformed tubules surrounded by interstitial

Table 2

Total number of PND 22 and 190 males with macroscopic reproductive effects after exposure to DEHP or DEHP and DEHA in combination from GD 7 to PND 17

	Control	DEHP (300 mg)	DEHP (750 mg)	DEHP + DEHA ^a
Number of PND 22 males	31	30	29	27
Number of PND 190 males	16	16	16	16
Small testis or lack of one testis		4	3	
Small/malformed epididymis		4	3	
Malformed seminal vesicles or spongy tissue in <i>m. levator ani/bulbocavernosus</i>		3		1
Cryptorchid testis			2	
Hypospadia ^b		1		
Abcesses in the uro-genital system				2

^a The mixture contained 750 mg DEHP and 400 mg DEHA/kg bw/day. Statistically significantly different compared to the control group ($p < 0.05$).

^b Only investigated in PND 22 males.

Table 3

Terminal body weight and organ weights at PND 22 after pre- and postnatally exposure to DEHP or DEHP and DEHA in combination

	Control	DEHP (300 mg/kg)	DEHP (750 mg/kg)	DEHP + DEHA ^a	<i>n</i>
Body weight (g)	45.8 ± 3.9	51.4 ± 5.9	45.0 ± 5.9	47.8 ± 6.2	30
Brain (g)	1.45 ± 0.055	1.47 ± 0.12	1.43 ± 0.075	1.45 ± 0.064	10
Liver (g)	1.73 ± 0.24	1.95 ± 0.30	1.73 ± 0.19	1.76 ± 0.19	10
Paired kidneys (mg)	512 ± 66	564 ± 89	512 ± 95	538 ± 81	10
Paired adrenals (mg)	22.0 ± 7.2	22.5 ± 6.9	19.5 ± 3.9	23.3 ± 6.4	10
Right testis (mg)	127 ± 13	134 ± 18	120 ± 23	120 ± 24	20
Left testis (mg)	128 ± 12	135 ± 18	117 ± 26	122 ± 24	30
Paired testes (mg)	256 ± 26	269 ± 37	235 ± 42*	240 ± 49	20
Left epididymis (mg)	25.9 ± 5.0	28.2 ± 7.8	21.3 ± 5.9	21.2 ± 4.2	10
Ventral prostate (mg)	29.9 ± 5.4	30.5 ± 5.8	23.8 ± 6.6	28.4 ± 5.5	10
Seminal vesicles (mg)	24.9 ± 7.3	30.6 ± 8.2	23.7 ± 5.3	26.8 ± 6.3	10
<i>M. levator ani/bulbocavernosus</i> (mg)	39.2 ± 7.3	42.3 ± 9.5	34.6 ± 7.6	37.8 ± 6.0	10

^a The mixture contained DEHP 750 mg/kg and DEHA 400 mg/kg. Data represents mean ± S.D. before exclusion of outliers.

* Statistically significantly different compared to the control group ($p < 0.05$).

Table 4

Terminal body weight and organ weights at PND 190 after pre- and postnatal exposure to DEHP or DEHP and DEHA in combination

	Control	DEHP (300 mg/kg)	DEHP (750 mg/kg)	DEHP + DEHA ^a
Body weight (g)	423 ± 33	421 ± 49	416 ± 29	428 ± 28
Brain (g)	1.95 ± 0.063	1.99 ± 0.13	2.06 ± 0.032	2.03 ± 0.069
Liver (g)	12.1 ± 1.3	12.3 ± 1.7	11.7 ± 0.99	12.6 ± 1.3
Paired kidneys (g)	2.17 ± 0.13	2.19 ± 0.22	2.23 ± 0.23	2.26 ± 0.25
Paired adrenals (mg)	47.5 ± 6.5	42.4 ± 10	45.9 ± 6.1	47.0 ± 11
Right testis (g) ^b	1.84 ± 0.15	1.65 ± 0.64 ^c	1.71 ± 0.47 ^c	1.88 ± 0.15
Left testis (g) ^b	1.91 ± 0.14	1.67 ± 0.56 ^c	1.84 ± 0.16	1.89 ± 0.17
Paired testis (g) ^b	3.76 ± 0.29	3.32 ± 1.1 ^c	3.55 ± 0.49	3.78 ± 0.32
Left epididymis (mg)	624 ± 46	556 ± 170 ^d	594 ± 76 ^d	633 ± 89
Ventral prostate (mg) ^b	584 ± 120	468 ± 120*	464 ± 88*	485 ± 98*
Seminal vesicles (g)	1.64 ± 0.31	1.39 ± 0.50 ^d	1.56 ± 0.29	1.40 ± 0.27
<i>M. levator ani/bulbocavernosus</i> (g) ^b	1.25 ± 0.21	1.06 ± 0.20*	1.02 ± 0.14*	1.05 ± 0.16*

^a The mixture contained DEHP 750 mg/kg and DEHA 400 mg/kg. Data represents mean ± S.D. before exclusion of outliers; $n = 16$ in all groups.

^b An interaction between age and dose was observed. Therefore the two age groups were analysed separately.

^c Two males from this group were severely affected by dose, considered as statistically outliers. When these were excluded from the analysis, no statistically significant differences were observed when compared to the control group.

^d One male from this group were severely affected by dose, considered as a statistically outlier. When this male was excluded from the analysis, no statistically significant differences were observed when compared to the control group.

* Statistically significantly different compared to the control group ($p < 0.05$).

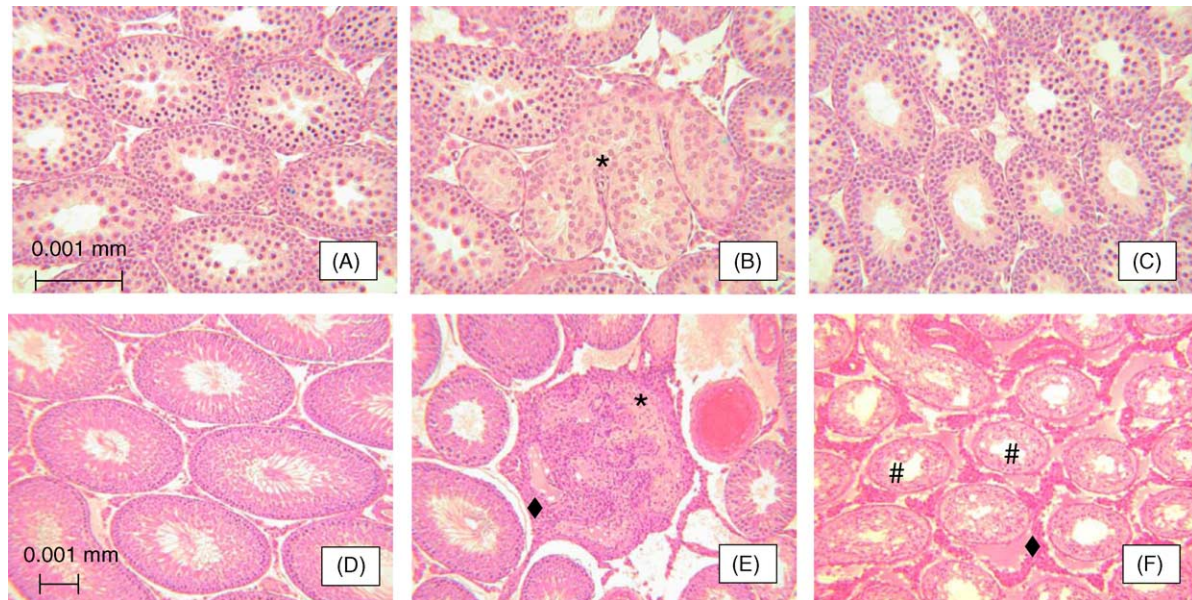


Fig. 4. Examples of testis effects in rats perinatally exposed to DEHP alone or DEHP in combination with DEHA. (A–C) 22-day-old males; (D–F) 190-day-old males. (A) and (D) are control testes; (B), (C), (E), and (F) are testes from males exposed to either DEHP (300 or 750 mg/kg) or DEHP (750 mg/kg) in combination with DEHA (400 mg/kg) – similar effects were seen in all three exposed groups. Foci containing malformed tubules (*) often surrounded by interstitial cell hyperplasia (◆) were observed at both PND 22 (B) and PND 190 (E) in all exposed groups but not in controls. A tendency to an increased incidence of seminiferous tubules with fewer spermatocytes and slightly disorganised seminiferous epithelium were observed in exposed males at PND 22 (C), but not at PND 190. However, 4 PND 190 males dosed with 300 mg/kg and 2 PND 190 males dosed with 750 mg/kg had severely atrophic seminiferous tubules (#) (end-stage tubules, where only Sertoli cells were left) (F). Staining: haematoxylin and eosin.

cell hyperplasia was found, (Fig. 4 B and E). These malformed tubules had disorganized seminiferous epithelium, decreased spermatogenesis, and tubular anastomoses. The incidence was lower at PND 190 compared to PND 22 (Table 5). Double staining with SMA and 3 β -HSD indicated that Leydig cells were present inside malformed tubules at PND 22 and 190 (Fig. 5 A–C).

At PND 22, tubules lacking spermatocytes and slightly disorganised tubules were observed in both control and dosed

testes although with a higher but non-significant incidence in the exposed groups (Table 5 and Fig. 4 C). These differences were not found in adults. At PND 190, severely atrophic testes with end-stage tubules (tubules with a total lack of germ cells and only Sertoli cells present) and diffuse Leydig cell hyperplasia were observed in four males exposed to 300 mg DEHP and two males dosed with 750 mg DEHP of which one was cryptorchid (Tables 2 and 5, Fig. 4 F). Four of these six males also had small epididymides.

Table 5

Histopathological effects in testes of PND 22 and 190 males after exposure to DEHP or DEHP in combination DEHA from GD 7 to PND 17

Number of affected males/total number of investigated males and in percent (%)	Control	DEHP (300 mg)	DEHP (750 mg)	DEHP + DEHA ^a
Foci with malformed tubules and interstitial cell hyperplasia				
PND 22	0/19 (0)	6/21 (29)*	5/18 (28)*	3/21 (14)
PND 190	0/14 (0)	3/16 (19)	2/15 (13)	5/16 (31)*
PND 22 + 190	0/33 (0)	9/37 (24)*	7/33 (21)*	6/37 (16)*
Slight disorganisation, lack of spermatocytes				
PND 22	5/19 (26)	9/21 (43)	9/18 (50)	8/21 (38)
PND 190	0/14 (0)	0/16 (0)	0/15 (0)	0/16 (0)
Severely atrophic testes				
PND 22	0/19 (0)	0/21 (0)	0/18 (0)	0/21 (0)
PND 190	0/14 (0)	4/16 (25)	2/15 (13)	0/16 (0)
Total number of males with effects on testes histology				
PND 22	5/19 (26)	14/21 (67)*	12/18 (67)*	10/21 (48)
PND 190	0/14 (0)	7/16 (44)*	4/15 (27)	5/16 (31)*
PND 22 + 190	5/33 (15)	21/37 (57)*	16/33 (48)*	15/37 (41)*

^a The mixture contained 750 mg DEHP and 400 mg DEHA/kg bw/day. In PND 22 males $n = 18$ –21. In PND 190 males $n = 14$ –16. In PND 22 about 10 testes were Bouin's fixed and the rest formalin fixed. In PND 190 males all testes were Bouin's fixed.

* $p < 5\%$ compared to the control group. Fisher's exact test.

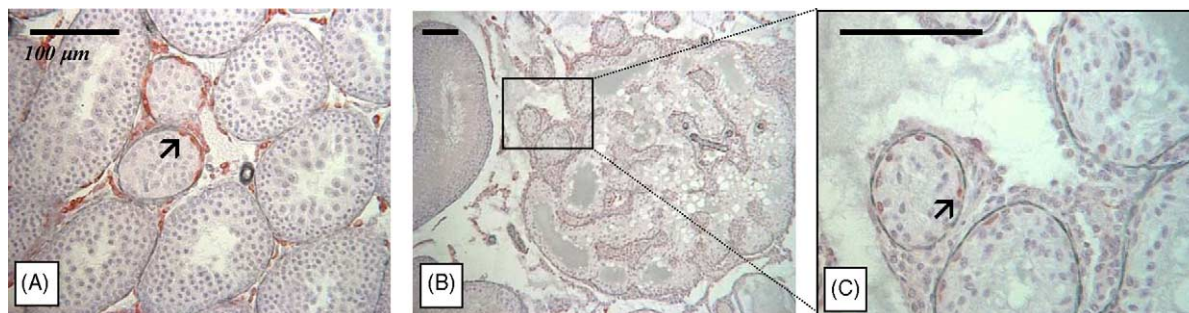


Fig. 5. Immunohistochemical characterization of dysgenic tubules in testes of young and adult male rats exposed to 750 mg DEHP/kg bw/day from GD 7 to PND 17. (A–C) Double immunostaining for α -smooth muscle actin (grey staining) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD, red staining) showing localization of Leydig cells inside seminiferous tubules in (A) 26-day-old rat testis; (B and C) 190-day-old rat testis, counterstained with Meyer's Hematoxylin. Control rat seminiferous tubules resemble the tubules neighboring dysgenic areas in these photos. Arrow indicates Leydig cells inside seminiferous tubules. Scalebar: 100 μ m.

At PND 22, no histopathological effects were observed in the epididymis, seminal vesicles, or the prostate, except for one male exposed to 750 mg DEHP, where a decrease in epithelium height from a cylindric to a cuboidal epithelium was observed in the proximal section of the epididymis. At PND 190, four males with testis effects (two males dosed with 300 mg DEHP and one male in each group dosed with either 750 mg DEHP or 750 mg DEHP in combination with 400 mg DEHA) had mild histopathological changes in the prostate consisting of a general lack of eosinophilic secrete in acini or a mild regional lymphocyte infiltration of the stromal tissue (data not shown).

When summarizing the numbers of males with any effects on the reproductive system, i.e. macroscopic and histological effects combined, the incidences were: 5 affected males of 47 males in the control group (10%), 22 affected of 46 males in the 300 mg DEHP group (48%), 17 affected of 45 males in the 750 mg DEHP group (38%), and 17 affected of 43 males in the 750 mg DEHP plus 400 mg DEHA group (40%). The total number of affected males was statistically significantly higher in all exposed groups compared to controls.

4. Discussion

The results of this study demonstrate that prenatal and lactational DEHP exposure can alter reproductive system development in male rats. Male offspring of dams exposed to 300 and 750 mg DEHP and a combination of 750 mg DEHP and 400 mg DEHA from GD 7 to PND 17 exhibited decreases in AGD, retention of nipples and decreases in the weight of ventral prostate and *m. levator ani/bulbocavernosus*. Furthermore, permanent alterations in testis histopathology were observed. Additionally, sperm counts, motility and velocity, as well as testis and epididymis weights were severely affected in a few animals.

4.1. Effects on dams and pup development

The lower maternal weight gain during gestation in dams from the combined dose group may not be an indication

of maternal toxicity, since maternal body weights after delivery were similar among groups. The low weight gain may rather be due to dams having fewer pups with a significantly decreased birth weight (Table 1). The number of pups per litter at birth was significantly decreased in litters exposed to DEHP in combination with DEHA and the post implantation loss was significantly increased. The high incidence of postnatal mortality in the combined group could be a combined effect of both DEHA and DEHP. We have previously found that DEHA induced weak developmental toxicity at 400 mg/kg bw/day and moderate developmental toxicity at 800 and 1200 mg/kg bw/day [21]. In the current study, it appeared that DEHP alone caused a trend towards a dose related increase in postnatal mortality, and a significant increase in postimplantation-perinatal loss in the 750 mg DEHP group was observed. Findings on reduced pup survival after in utero phthalate exposure have previously been reported for DEHP [14] and DBP [13] at doses of 750 mg/kg bw/day.

The AGD was significantly decreased in all exposed groups in a dose-related manner, also when correcting for body weight, which is in agreement with studies on DEHP by Moore et al. [14] and Parks et al. [18]. The effect on AGD was most severe in the group receiving the combined dose of DEHP and DEHA, though there was no significant difference in AGD between the 750 mg DEHP dose group and the combined dose group. The development of external genitals is affected by dihydrotestosterone (DHT). The reduction of AGD may be secondary to low DHT levels caused by a reduction in prenatal testosterone production, as shown in studies on 19–21-day-old foetal males exposed to DEHP in utero [18,19].

More areolas/nipples were present at PND 13 in males in all three exposed groups compared to the control group. The distribution in male offspring in the 300 mg DEHP dose group and in the 750 mg DEHP plus 400 mg DEHA dose group were almost similar (Fig. 1). The lower number of areolas/nipples in the combined exposure group compared to the 750 mg DEHP group may be explained by the high postnatal mortality in the combination group, as the most

severely affected males may be dead at PND 13, the day when number of nipples is counted.

4.2. Effects on sperm quality

Effects on sperm counts, motility and velocity parameters were observed in 3 males exposed to 300 mg/kg DEHP and 2 males exposed to 750 mg/kg DEHP and these 5 males all had atrophic testes. The fewer living sperm cells probably account for the decreased percentage of motile sperm. Effects of DEHP on sperm count have previously been demonstrated in other studies where animals were exposed either perinatally [14,22] or in adulthood [15,23]. These findings may either be a result of the effect of DEHP on Sertoli cells that nurse the developing spermatozoa, or on the epididymides, where the maturation of sperm cells takes place.

4.3. Histological effects

At PND 22, slightly disorganised tubules and lack of spermatocytes was observed in both controls and dosed males (Fig. 4 C). These findings probably reflect variability among animals in the timing of the first spermatogenic cycle. The incidence was increased in the dosed groups compared to the control group and even though this increase was not statistically significant, it may reflect a delay in spermatogenesis in these young males, caused by the DEHP induced testes effects observed in late gestation (see accompanying paper [24]). Correspondingly, levels of serum inhibin B, a marker of Sertoli cell function, were significantly reduced at PND 22 in these males, possibly reflecting delayed Sertoli cell maturation as reported in Borch et al. [19]. The statistically significant decrease in paired testis weight in the high dose DEHP group at PND 22 supports this presumed delay in the progress of testicular maturation.

Six adult males from the DEHP groups had severely atrophic testes with degeneration of the seminiferous epithelium. The highly abundant effects described in fetuses [24] are in contrast to the very few affected adult testes. This gives rise to speculations of why some testes appear to recover, and others continue an inappropriate development. It is well known that fetal Leydig cells are affected by phthalates in addition to fetal Sertoli cell/germ cell interactions [18,20,25]. Early Sertoli cell dysfunction may cause the later spermatogenic failure, and indeed serum inhibin B is affected in prepubertal males exposed to DEHP as well as in adult males with atrophic testes. It is possible that Sertoli cells in the most affected testes in prepuberty never mature completely, leading to the observed dysfunction of spermatogenesis and testicular atrophy in adults. On the other hand, the testicular atrophy may also be secondary to effects on the epididymis, as fetal Leydig cell dysfunction resulting in reduced testosterone production will likely affect the development of androgen dependent organs. This is demonstrated by the observed atrophy of a few epididymides, in addition to the statistically significant reduction of the weights of the ventral prostate

and *m. levator ani/bulbocavernosus* in all exposed groups at PND 190 (Table 4).

Interestingly, Barlow and Foster have recently studied the progress of effect of DBP from foetal age until adulthood after in utero exposure. They observed that DBP-induced testicular lesions progressed in severity from PND 45 to 70, and suggested that the testicular atrophy induced by DBP was secondary to epididymal obstruction [24]. Also, in utero exposure to linuron induced obstruction of testicular fluid outflow following epididymal malformations causing testicular atrophy that was much more profound at PND 56 compared to PND 35 [26]. In the current study, four of the six males with atrophic testes also had small epididymides. Unfortunately, epididymal histology could be evaluated in only one of the small epididymides, as the remaining epididymides were sampled for semen analyses. In this epididymis, the ductular lumen was decreased and contained no sperm. In the current study, none of the examined testes had dilated seminiferous tubules as seen by Barlow & Foster in prenatally DBP exposed rats at PND 45 but not at PND 70. If dilation of seminiferous tubules was indeed an early step in the observed seminiferous tubules degeneration, this would likely not be seen at our examination at PND 190. The hypothesis of epididymal obstruction is confirmed by the examination at PND 22, in which we observed one epididymis with dilated ductular cross sections with flattened epithelium.

Persisting Leydig cell dysfunction was generally not seen in the current study, where testosterone levels were normal in prepubertal animals and most adults, but low in males with atrophic testes. The observed Leydig cell hyperplasia in the atrophic testes may be caused by elevated LH levels as an attempt to compensate for the low testosterone levels.

Areas of malformed tubules surrounded by focal areas of interstitial cell hyperplasia were found in the otherwise normal testes of males dosed with DEHP or with DEHP in combination with DEHA. The incidence was lower at PND 190 compared to PND 22, which is likely due to the dysgenic tubules becoming surrounded by an increasing volume of normally growing testis tissue during puberty and adulthood. Leydig cells were found inside the dysgenic tubules, as also reported by Fisher et al. in a study on neonatal males exposed in utero to DBP [27]. As discussed in the accompanying paper [24], the dysgenic areas probably originate from foetal or early postnatal life.

4.4. Are the antiandrogenic effects of DEHP modulated by DEHA?

Based on the result in this study, no modulating effect of DEHA on the antiandrogenic effects of DEHP was observed. However, the litter size at birth was significantly decreased and postnatal pup mortality increased in the combination group only. These effects are likely reflecting the developmental toxicity of DEHA as seen at 800 mg/kg DEHA in our previous dose-response study [27]. By combining DEHA with DEHP, DEHP seems to advance this developmental

toxicity of DEHA. Including a dose group receiving DEHA alone in our study design would have strengthened the conclusions in the current study. Even though the effect on AGD seemed more pronounced in the combined dose group, the difference to the 750 mg DEHP group was not statistically significant. In addition, the other endpoint for antiandrogenicity, nipple retention, was even less affected in the combination group compared to the high dose of DEHP, further indicating that DEHA does not increase the antiandrogenic effect of DEHP. These findings confirm previous studies in our laboratory where perinatal exposure up to 1200 mg DEHA/kg bw/day did not reduce the AGD in male offspring [21]. Generally, DEHA does not increase the incidence or severity of effects on organ weights, sperm analysis and histopathology.

Variability in responsiveness from animal to animal seen in this study was rather remarkable, as some individuals in the exposed dose groups were severely affected, while others were more or less unaffected. Also Gray et al. reported large animal-to-animal variation after in utero exposure to DEHP [11]. We examined all males from each litter macroscopically, but we may have reduced the chance of seeing all effects by using each type of tissue for many parameters (histopathology, semen quality, hormone analyses). The affected animals in our study could thus represent a minimum of effects.

In summary, adverse effects of DEHP as shown by others have been confirmed in this study. Testicular atrophy, reduced weights of reproductive organs, reduced anogenital distance and an increased number of nipples/areolas were found in perinatally exposed males. Dysgenic tubules containing Leydig cells were found within otherwise normal DEHP-exposed testes. The litter size was decreased and postnatal mortality was increased in the combination group only, which is likely a combined effect of DEHP and DEHA. However, no combination effect was seen with respect to antiandrogenic effects, as males receiving DEHP in combination with DEHA did not exhibit more pronounced effects in the reproductive system than males receiving DEHP alone.

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Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis

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Abstract

Diethylhexyl phthalate (DEHP) is widely used as a plasticizer in consumer products and is known to disturb the development of the male reproductive system in rats. The mechanisms by which DEHP exerts these effects are not yet fully elucidated, though some of the effects are related to reduced fetal testosterone production.

The present study investigated the effects of four different doses of DEHP on fetal testicular histopathology, testosterone production and expression of proteins and genes involved in steroid synthesis in fetal testes.

Pregnant Wistar rats were gavaged from GD 7 to 21 with vehicle, 10, 30, 100 or 300 mg/kg bw/day of DEHP. In male fetuses examined at GD 21, testicular testosterone production *ex vivo* and testicular testosterone levels were reduced significantly at the highest dose. Histopathological effects on gonocytes were observed at 100 and 300 mg/kg bw/day, whereas Leydig cell effects were mainly seen at 300 mg/kg bw/day. Quantitative RT-PCR revealed reduced testicular mRNA expression of the steroidogenesis related factors SR-B1, StAR, PBR and P450scc. Additionally, we observed reduced mRNA expression of the nuclear receptor SF-1, which regulates certain steps in steroid synthesis, and reduced expression of the cryptorchidism-associated Insl-3.

Immunohistochemistry showed clear reductions of StAR, PBR, P450scc and PPAR γ protein levels in fetal Leydig cells, indicating that DEHP affects regulation of certain steps in cholesterol transport and steroid synthesis. The suppression of testosterone levels observed in phthalate-exposed fetal rats was likely caused by the low expression of these receptors and enzymes involved in steroidogenesis. It is conceivable that the observed effects of DEHP on the expression of nuclear receptors SF-1 and PPAR γ are involved in the downregulation of steroidogenic factors and testosterone levels and thereby underlie the disturbed development of the male reproductive system.

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1. Introduction

It has been suggested that the observed increasing incidences of testicular cancer, hypospadias, crypt-

orchidism and reduced semen quality in recent years may be caused by endocrine disrupting environmental compounds with adverse effects on male reproduction (Skakkebaek *et al.*, 2001). Among the chemicals suspected of causing these effects in humans are the abundantly used phthalates found in plastics, paints and other materials. Human studies have indicated associations between maternal phthalate exposure and altered

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reproductive development in their infant sons (Swan et al., 2005). Some of the most commonly used phthalates are known to induce adverse effects in the reproductive system of male rats. Perinatal phthalate exposure leads to anti-androgenic effects such as reduced anogenital distance (AGD), retained nipples, hypospadias, undescended testes, epididymal agenesis and low sperm counts in male rats (Barlow and Foster, 2003; Jarfelt et al., 2005).

The mechanisms by which phthalates exert these effects are not yet fully elucidated. Some of the anti-androgenic effects seen after pre- or perinatal exposure of rats are likely caused by the observed reduction in testosterone production in late gestation. Normally, testosterone levels in male rats increase from gestational day (GD) 17 and peak at GD 20 (Parks et al., 2000). DEHP, di-*n*-butyl phthalate (DBP), diisononyl phthalate (DINP) and diisobutyl phthalate (DIBP) have been found to reduce testosterone levels between GD 17 and post-natal day (PND) 2 (Borch et al., 2004; Parks et al., 2000; Borch et al., 2006). Studies on DBP have shown reduction of fetal testicular mRNA and protein levels of a number of factors related to testosterone production. The factors affected by DBP include scavenger receptor B-1 (SR-B1) responsible for cholesterol uptake into Leydig cells, steroidogenic acute regulated protein (StAR) and peripheral benzodiazepine receptor (PBR) both involved in mitochondrial cholesterol uptake, and the steroid-converting enzymes P450 side chain cleavage (P450_{scc}) and P450_{c17} (Barlow et al., 2003; Lehmann et al., 2004; Thompson et al., 2004). However, the mechanism behind the downregulation of these factors is not known.

Whereas focus has so far been aimed at elucidating the mechanism of action of DBP, similar studies on the mechanism of action of DEHP or other phthalates have not been published so far. Liu et al. (2005) showed by microarray analyses that DBP, DEHP, benzylbutyl phthalate and dipentyl phthalate evoke a similar pattern of changes in gene expression, indicating that these phthalates target the same pathways in male reproductive development.

In the present study, we examined the effects of DEHP on mRNA and protein levels of factors related to the synthesis of steroids (SR-B1, StAR, PBR, P450_{scc}, P450_{c17}) at GD 21 in the testes of fetal rats exposed to doses from 10 to 300 mg/kg bw/day of DEHP. We have previously observed reduced fetal testosterone levels at 300 and 750 mg DEHP/kg bw/day (Borch et al., 2004) and aimed to examine effects on steroid synthesis as well as histopathology at lower dose levels.

Additionally, we examined the expression of Leydig cell product insulin-like factor-3 (Insl-3), which is

involved in testicular descent, and two factors essential for fetal Leydig cell differentiation, the Sertoli cell product Desert hedgehog (Dhh) and its receptor Patched-1 (Ptc-1) in Leydig cells (Yao and Capel, 2002). A study on prenatal flutamide exposure of rats showed downregulation of testicular Dhh, Ptc-1 as well as Insl-3 expression at GD 18 and GD 20 (Brokken et al., in preparation). It may thus be speculated that impaired Dhh and Ptc-1 function may underlie reduced expression of Insl-3 and steroidogenesis related factors.

The expression of the nuclear receptors steroidogenic factor-1 (SF-1), and peroxisome proliferator activated receptors- α and - γ (PPAR α and PPAR γ) was also examined. SF-1 is known to regulate the expression of genes involved in steroidogenesis, and PPARs are nuclear receptors that among many other functions regulate genes involved in cholesterol uptake and transport (Xie et al., 2002). A role for PPARs in the testicular toxicity of phthalates may be speculated, as the most testis toxic phthalates are strong PPAR activators (reviewed by Corton and Lapinskas, 2005). Several PPAR α or PPAR γ activating chemicals are known to affect steroid production in testes and ovaries of rats and humans in vitro and in vivo. PPAR agonists such as phthalates, ammonium perfluorooctanoate, clofibrate and WY14,643 have been found to reduce testosterone production in primary Leydig cell culture and in adult rats (Biegel et al., 1995; Csaba et al., 1995; Gazouli et al., 2002; Liu et al., 1996). Furthermore, the two PPAR γ activating thiazolidinedione drugs troglitazone and rosiglitazone reduced testosterone levels in humans (Vierhapper et al., 2003; Bloomgarden et al., 2001). If the effects of phthalates in rat testes are related to PPARs, this mechanism of action may thus be highly relevant to humans.

Testicular gene and protein expression of a number of factors related to steroid synthesis and/or Leydig cell function were examined in order to compare the effects of DEHP with those observed with DBP in previous studies and to elaborate further on the mechanisms of actions of DEHP.

2. Materials and methods

2.1. Test compounds

DEHP, di(2-ethylhexyl) phthalate, CAS no. 117-81-7, purity 99%, was from Aldrich 20,115-4.

2.2. Animals and dosing

Forty time-mated Wistar rats (HanTac:WH, Taconic M&B, Denmark, body weight approx. 200 g) were supplied at day 3

of pregnancy. The day following mating was designated GD 1. The dams were randomized into five groups of eight with similar body weight distributions and housed in pairs until GD 21 under standard conditions. Semi-transparent plastic cages with pinewood bedding were situated in an animal room with controlled environmental conditions (12 h light–dark cycles with light starting at 9 p.m., light intensity 500 lx, temperature $21 \pm 2^\circ\text{C}$, humidity $50 \pm 5\%$, ventilation 8 air changes/h). Food (Altromin Standard diet 1324) and tap water were provided *ad libitum*. The animals were dosed with vehicle (corn oil), 10, 30, 100 or 300 mg DEHP/kg bw/day from GD 7 to GD 21. Animals were inspected for general toxicity twice daily. On GD 21, dams were anesthetized in CO_2/O_2 and decapitated, and fetuses were removed and decapitated.

Testes were removed and sampled for gene expression, histopathology, measurement of testosterone production *ex vivo*, or measurement of testosterone content. One or two testes per litter were placed in RNAlater (QIAGEN) and kept at -20°C until extraction of RNA and cDNA production for use in real-time RT-PCR. One or two testes per litter were placed in Bouin's fixative, and one testis per litter was placed in neutral buffered formaldehyde for histopathology and immunohistochemistry. For measurement of testosterone production *ex vivo*, one testis per litter was placed in 0.5 ml ice-cold Dulbecco's modified eagle medium (DMEM)/F12 with 15 mM HEPES, 365 mg/l L-glutamine plus 0.1% bovine serum albumin and 0.1 g/l gentamicin. The testes were incubated at 37°C for 5 h, were then placed on ice, centrifuged at $4000 \times g$ for 10 min, and the supernatant was collected and stored at -80°C until analysis of testosterone content. One testis per litter was placed in an empty tube and immediately frozen in liquid nitrogen and stored at -80°C until analysis. Testosterone was extracted by two consecutive diethylether extraction procedures and stored at -80°C until analysis.

Trunk blood from all males was collected with heparinized microfuge tubes and kept on ice. Pools of blood from each group was centrifuged at $4000 \times g$ for 10 min at 4°C , and kept at -80°C until analysis of testosterone content. Steroids were extracted from plasma samples by solid-phase extraction using IST Isolute C18 SPE columns of 100 mg. Testosterone content in incubation media, testes and plasma was analyzed with a Delfia time-resolved fluoroimmunoassay (Perkin-Elmer, Wallac Oy, Turku, Finland). Plasma cholesterol was measured on one pool of plasma from each dose group using a Roche/Hitachi analyzer system.

2.3. Gene expression

mRNA expression was measured in 1 or 2 animals per litter, 10 animals per group. Two testes from the same animal were pooled and homogenized in RLT buffer from the RNeasy Mini kit (QIAGEN), and total RNA was isolated using RNeasy-mini kit from QIAGEN following the supplier's protocol for animal tissues. The quantity and quality of purified RNA was evaluated by spectroscopy. cDNA was produced by Omniscript kit

(QIAGEN) from 1 μg total RNA using T16 primer and 18S rRNA specific oligos.

Expression of SR-B1, StAR, P450scc, P450c17, PBR, PPAR γ and PPAR α was analyzed by quantitative RT-PCR on ABI PRISM 7900 HT (Applied Biosystems) by standard TaqMan technology using fluorescence monitoring. TaqMan probes contain a reporter dye at the 5'-end and a quencher dye at the 3'-end is cleaved during the PCR, and fluorescence from the reporter dye is detected. Quantitative results were obtained by the cycle threshold value where a signal rises above background level. Rat specific primers and TaqMan probes from Applied Biosystems are listed in Table 1. Optimal concentrations were determined for each set of probe and primers. For each sample, 2 μl cDNA (2.5 ng/ μl) was amplified under universal thermal cycling parameters (Applied Biosystems) using TaqMan Universal PCR master mix (Applied Biosystems) in a total reaction volume of 20 μl . For each sample three separate measurements were performed. A fourth measurement was made when intra-assay variation was above $>10\%$. Sample signals were quantified from standard curves and expression levels of each gene were normalized to the level of the housekeeping gene 18S rRNA.

Expression of Desert hedgehog (Dhh), Patched 1 (Ptc-1), Insl-3, SF-1 and Ribosomal protein S26 (S26) was measured by quantitative PCR (QuantiTect SYBR-Green PCR Kit, Qiagen) according to the manufacturer's instructions by using the DNA Engine Opticon system (MJ Research Inc., Waltham, MA) with continuous fluorescence detection. Primers for Insl-3, SF-1, Dhh, Ptc-1 and S26 are listed in Table 1. About 2 μl cDNA per sample was used for the amplification. Single measurements were made for all dose groups. As the expression of Insl-3 and SF-1 appeared to be affected by the highest dose of DEHP, Insl-3, SF-1 and S26 were subsequently repeated in triplicates for the control and high dose group. Expression levels of Insl-3 and SF-1 were quantified from standard curves and normalized to the level of the housekeeping gene S26.

2.4. Histopathology and immunohistochemistry

Sections from testes fixed in Bouin's fixative were stained with haematoxylin and eosin for histopathological evaluation blinded to the observer. Immunohistochemistry was performed on one section per testis. Following microwave pretreatment for 2×5 min in either citrate or TEG buffer, sections were blocked for endogenous peroxidase activity in 3% H_2O_2 in PBS, and blocked in 1% bovine serum albumin in PBS. Sections were incubated over night at 4°C with the following rabbit polyclonal antibodies: StAR antibody (PA1-560, Affinity Bioreagents, Golden, CO, 1:1000), P450scc antibody (AB 1244, Chemicon, Temecula, CA, 1:50,000), β -hydroxysteroid dehydrogenase (β -HSD) antibody (a kind gift from Dr. I. Mason, Edinburgh, UK, 1:4000), PPAR γ antibody (Cell Signaling, Beverly, MA, 1:100), PBR antibody (SantaCruz, CA, 1:200). Sections were then incubated for 30 min with secondary antibody (Anti-rabbit EnVision+,

Table 1
Rat specific primer sets and TaqMan probes for quantitative RT-PCR analyses

18S	
TaqMan probe	6-FAM-ACC GGC GCA AGA CGA ACC AGA G-TAMRA
Forward primer	GCC GCT AGA GGT GAA ATT CTT G
Reverse primer	GAA AAC ATT CTT GGC AAA TGC TT
SR-B1	
TaqMan probe	6-FAM-AAA GCA TTT CTC CTG GCT GCG CAG-TAMRA
Forward primer	TCT GGT GCC CAT CAT TTA CCA
Reverse primer	AGC CCT TTT TAC TAC CAC TCC AAA
StAR	
TaqMan probe	6-FAM-CTG ACT CCT CTA ACT CCT GTC TGC CTA CAT GGT-TAMRA
Forward primer	CCC TTG TTT GAA AAG GTC AAG TG
Reverse primer	TGA AAC GGG AAT GCT GTA GCT
P450scc	
TaqMan probe	6-FAM-CCT TTA TGA AAT GGC ACA CAA CTT GAA GGT ACA-TAMRA
Forward primer	ACG ACC TCC ATG ACT CTG CAA T
Reverse primer	CTT CAG CCC GCA GCA TCT
P450c17	
TaqMan probe	6-FAM-CGT CAA CCA TGG GAA TAT GTC CAC CAG A-TAMRA
Forward primer	GCC ACG GGC GAC AGA A
Reverse primer	CCA AGC CTT TGT TGG GAA AA
PPAR α	
TaqMan probe	6-FAM-CAA TCC GCC TTT TGT C-MGB
Forward primer	TCA TAC TCG CAG GAA AGA CTA GCA
Reverse primer	GCA CAA GGT CTC CAT GTC ATG T
PPAR γ	
TaqMan probe	6-FAM-TAT GAC CTG AAG CTC CAA GAA TAC CAA AGT GC-TAMRA
Forward primer	TGA CCC AAT GGT TGC TGA TTA C
Reverse primer	GGG ACG CAG GCT CTA CTT TG
PBR	
TaqMan probe	6-FAM-AAA GCC CAG CCC ATC T-MGB
Forward primer	TGG TTC CCT TGG GTC TCT ACA CT
Reverse primer	CAC CCC ACT GAC AAG CAT GA
S26	
Forward primer	AAG GAG AAA CAA CGG TCG TG
Reverse primer	GCA GGT CTG AAT CGT GGT G
Insl-3	
Forward primer	ACG CAG CCT GTG GAC ACC
Reverse primer	CAA TCC GGG GGT GTT TCA TT
SF-1	
Forward primer	GCT TGT CTC AAG TTC CTC ATC CTC
Reverse primer	CTC GTT GCC CAA ATG CTT AT
Dhh	
Forward primer	AAG CAA CTT GTG CCT CTG CT
Reverse primer	TCG TCC CAA CCT TCA GTC AC
Ptc-1	
Forward primer	TAC GTG GAG GTG GTT CAT CA
Reverse primer	AAC ACC AAG GGC AAG AAA TG

DAKO, Glostrup, Denmark), stained in diaminobenzidine (DAB+ Substrate Chromogen System, DAKO, Glostrup, Denmark) or 3-amino-9-ethylcarbazole (AEC, Labvision, CA), and counterstained in Meyer's hematoxylin.

2.5. Statistics

Non-processed and logarithmically transformed hormone and gene expression data were examined for normal distribu-

tion and homogeneity of variance. Normally distributed and variance homogeneous data were analyzed by ANOVA using single animal data. For datasets including more than one animal per litter, the litter was the statistical unit and included in the analysis of variance as an independent, random factor nested within treatment group (SAS Analyst, Mixed Procedure, SAS version 8, SAS Institute Inc., Cary, NC, USA). Dunnett's tests were performed to determine differences between treated groups and control group means. Asterisks indicate a statistically significant difference compared to controls, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3. Results

Testicular histopathology was examined in one or two testes per litter from 21-day-old fetal rats exposed to 0, 10, 30, 100, or 300 mg DEHP/kg bw/day from GD 7 to GD 21. At the two highest doses germ cells were centrally located, the number of gonocytes appeared to be increased, and multinucleated germ cells were present (Fig. 1). At the highest dose level, vacuolisation of Sertoli cell and clusters of spindle shaped Leydig cells were observed.

We measured testosterone levels as well as testicular gene and protein expression of factors involved in testosterone synthesis and Leydig cell function. Testosterone levels were analysed in plasma as well as in testes and in addition, the ability of the fetal testis to produce testosterone *ex vivo* was measured. Testicular testosterone content and testicular testosterone production *ex vivo* were reduced by DEHP. For both parameters, the effect was statistically significant at the highest dose compared to controls (Fig. 2A and B). Similarly, a clear tendency towards reduced plasma testosterone levels was found (Fig. 2C). However, as blood from all male fetuses in each dose group had been pooled, an average level was measured and no statistical analysis was possible.

In order to examine the role of cholesterol availability for testicular testosterone synthesis, cholesterol levels were measured in pools of plasma from all male fetuses in each dose group. No differences between groups were observed (data not shown).

The effect of DEHP on factors involved in steroid synthesis was examined by quantitative analysis of mRNA expression and by immunohistochemistry on GD 21 testes. The gene expression of SR-B1, StAR, PBR and P450scc were reduced dose-dependently. The effect was statistically significant for StAR at the two highest doses and for SR-B1, PBR and P450scc at the highest dose (Fig. 3A–D). Similarly, there was a tendency towards lower transcript levels of P450c17 (Fig. 3E).

Immunohistochemical staining of testis sections from the highest dose group were compared to controls. Cor-

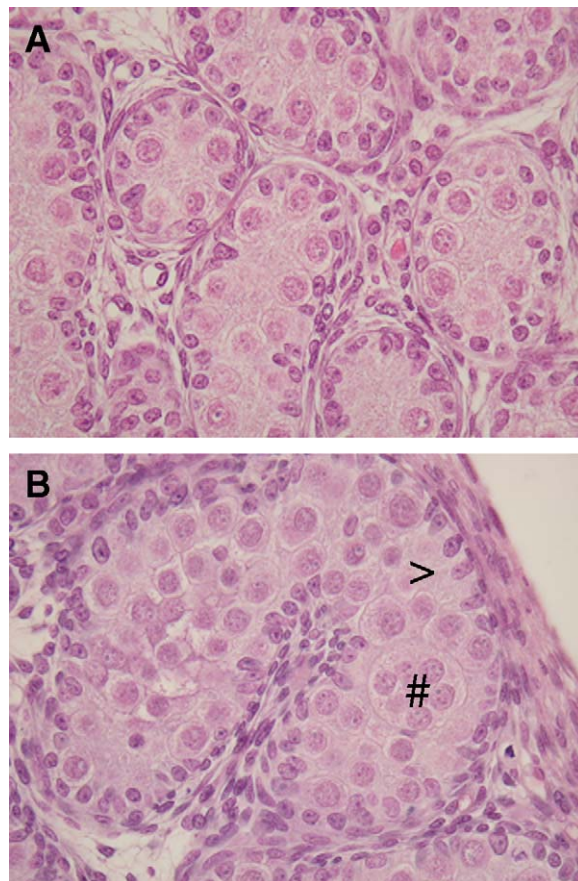


Fig. 1. Testicular histopathology at GD 21 in male rat fetuses exposed to 0 or 300 mg DEHP/kg bw/day from GD 7 to GD 21. Testes of DEHP-exposed animals (B) had a larger number of gonocytes compared to controls (A). Gonocytes were located centrally in the seminiferous tubules due to vacuolization of Sertoli cell cytoplasm (>). Multinucleated gonocytes (#) were present in testes of DEHP-exposed animals. Original magnification 40 \times .

responding to the gene expression findings, the protein expression of StAR, PBR and P450scc protein in Leydig cells was reduced by DEHP (Fig. 4). No change in 3 β -HSD staining intensity was observed (not shown). Collectively, these effects on mRNA and protein expression indicate that a general downregulation of the steroid synthesis pathway leads to the observed low testosterone levels.

Additionally, we examined the mRNA expression of other factors involved in Leydig cell function. SF-1, which regulates the expression of genes involved in steroidogenesis, and Insl-3, which is involved in testicular descent, were both significantly reduced in the highest dose group compared to controls (Fig. 5).

As the nuclear receptors PPAR α and PPAR γ regulate genes involved in cholesterol uptake and transport, we

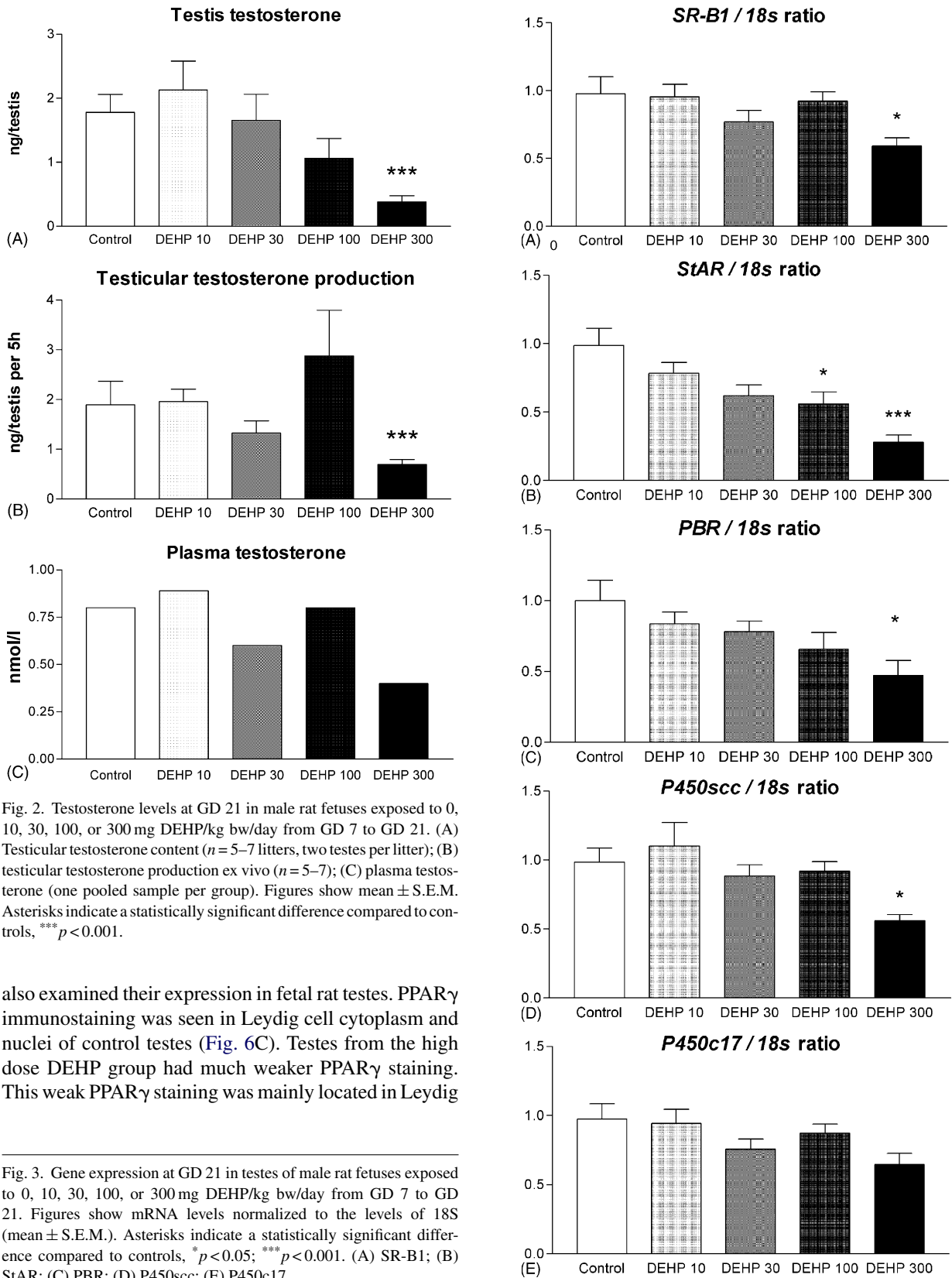


Fig. 2. Testosterone levels at GD 21 in male rat fetuses exposed to 0, 10, 30, 100, or 300 mg DEHP/kg bw/day from GD 7 to GD 21. (A) Testicular testosterone content ($n = 5-7$ litters, two testes per litter); (B) testicular testosterone production ex vivo ($n = 5-7$); (C) plasma testosterone (one pooled sample per group). Figures show mean \pm S.E.M. Asterisks indicate a statistically significant difference compared to controls, *** $p < 0.001$.

also examined their expression in fetal rat testes. PPAR γ immunostaining was seen in Leydig cell cytoplasm and nuclei of control testes (Fig. 6C). Testes from the high dose DEHP group had much weaker PPAR γ staining. This weak PPAR γ staining was mainly located in Leydig

Fig. 3. Gene expression at GD 21 in testes of male rat fetuses exposed to 0, 10, 30, 100, or 300 mg DEHP/kg bw/day from GD 7 to GD 21. Figures show mRNA levels normalized to the levels of 18S (mean \pm S.E.M.). Asterisks indicate a statistically significant difference compared to controls, * $p < 0.05$; *** $p < 0.001$. (A) SR-B1; (B) StAR; (C) PBR; (D) P450scc; (E) P450c17.

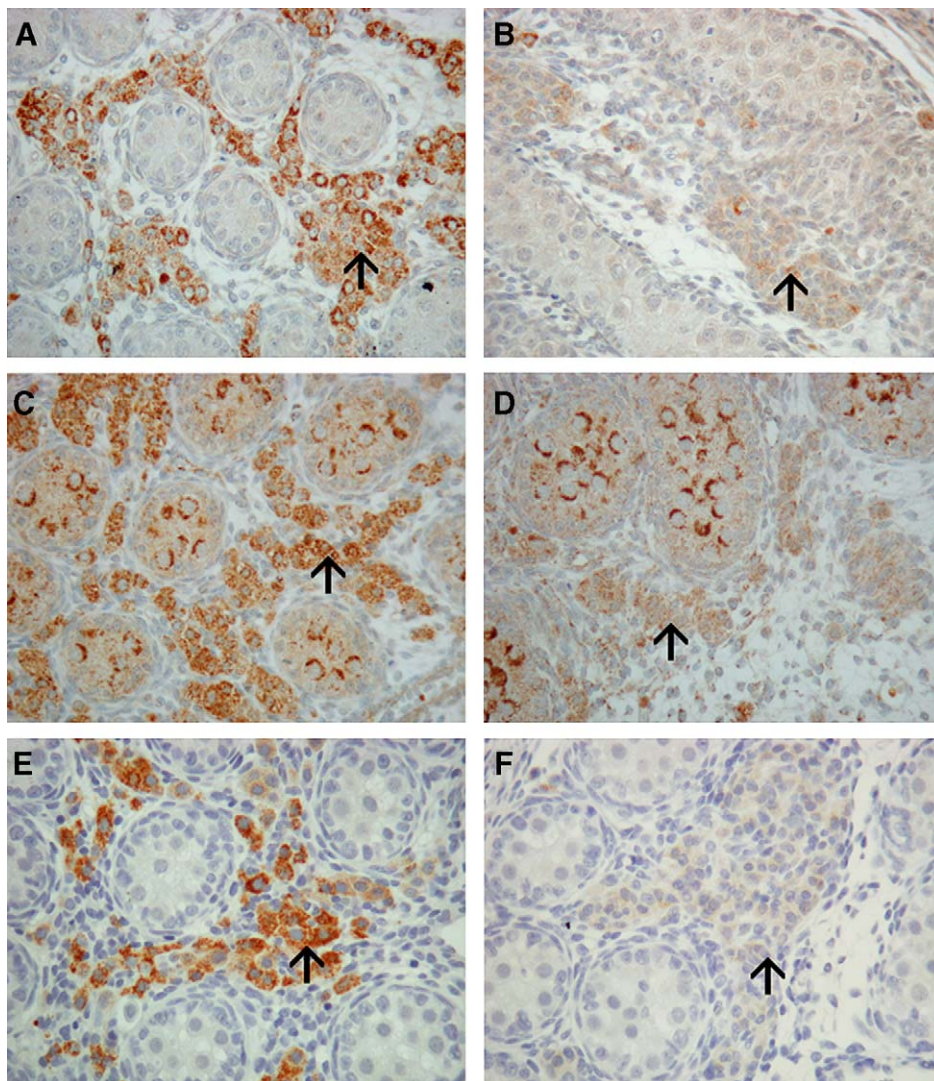


Fig. 4. Immunohistochemical staining at GD 21 of testes from male rat fetuses exposed to 0 or 300 mg DEHP/kg bw/day from GD 7 to GD 21. The intensity of staining in Leydig cells (\uparrow) for StAR (A and B), PBR (C and D), and P450scc (E and F) is reduced in DEHP-exposed animals (B, D and F) compared to controls (A, C and E).

cell nuclei and almost absent in the cytoplasm (Fig. 6D). The effect of DEHP on PPAR γ protein expression was not matched by gene expression results, although PPAR γ mRNA levels appeared slightly reduced at the highest dose compared to controls (Fig. 6A). PPAR α mRNA levels were not altered by DEHP treatment (Fig. 6B).

In order to give an overview of the observed findings, the DEHP-induced effects on mRNA and protein expression is shown in Table 2 and for comparison it is indicated which effects have previously been described for DBP (Barlow et al., 2003; Lehmann et al., 2004; Thompson et al., 2004).

4. Discussion

The current study showed reduced testicular testosterone production *ex vivo* and testicular testosterone levels in rat fetuses exposed to DEHP in utero. The testosterone suppressing effect was significant at 300 mg DEHP/kg bw/day, a dose level that induces atrophy of testes and epididymides, reduced anogenital distance and an increased number of nipples in male rats exposed in utero (Jarfelt et al., 2005). Recent studies have shown reduced anogenital distance and an increased number of nipples at doses from 10 mg DEHP/kg bw/day (Hass et al., 2004).

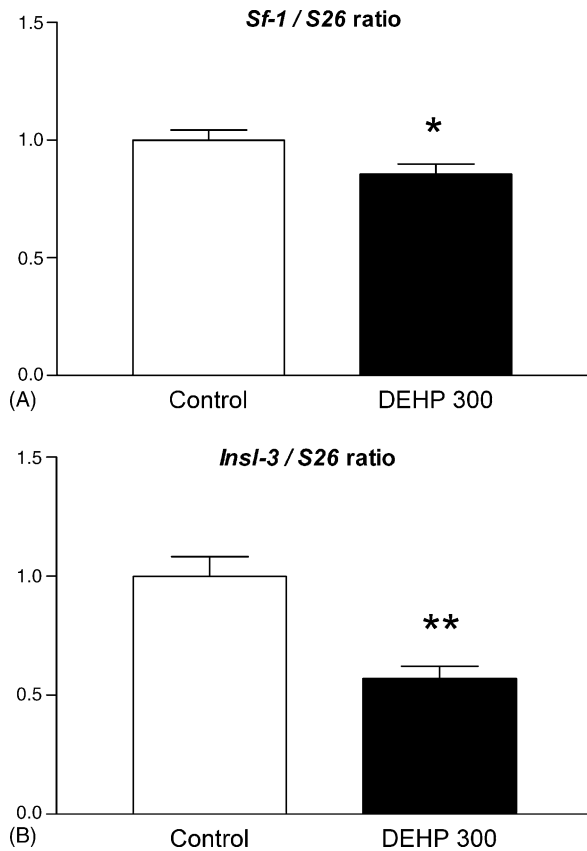


Fig. 5. Gene expression at GD 21 in testes of male rat fetuses exposed to 0 or 300 mg DEHP/kg bw/day from GD 7 to GD 21. Figures show mRNA levels normalized to the levels of S26 (mean \pm S.E.M.). Asterisks indicate a statistically significant difference compared to controls, * $p < 0.05$; ** $p < 0.01$. (A) SF-1; (B) Insl-3.

Histopathological effects on gonocytes were seen at the two highest dose levels, 100 and 300 mg DEHP/kg bw/day. Leydig cell alterations were observed at the highest dose, at which testosterone levels and steroidogenic factors were also affected. The observation of effects on gonocytes at lower doses may indicate that DEHP disrupts pathways affecting gonocyte development at doses which do not affect Leydig cell function.

Gene and/or protein expression of the steroidogenic factors SR-B1, StAR and P450scc were reduced in testes of DEHP-exposed animals, as summarized in Table 2. These effects have previously been described for DBP, but not for any other phthalate (Barlow et al., 2003; Lehmann et al., 2004; Thompson et al., 2004). Furthermore, this is the first paper to describe reduced expression of SF-1 and PBR and reduced protein levels of PPAR γ in fetal testes of rats exposed to any phthalate. As SF-1 and PPARs regulate certain steps in cholesterol transport and steroid synthesis, it may be hypothesized

Table 2

Effects of gestational exposure to DEHP (300 mg/kg bw/day) on testicular mRNA or protein expression of factors involved in fetal steroidogenesis or testicular descent in male rat fetuses

	Effect of DEHP on mRNA expression measured by quantitative RT-PCR	Effect of DEHP on protein expression assessed by immunohistochemistry
SR-B1	↓ (↓)	NA (↓)
StAR	↓ (↓)	↓ (↓)
PBR	↓ (↑)	↓ (↓)
P450scc	↓ (↓)	↓ (↓)
3 β -HSD	NA (↓)	↔
P450c17	↔ (↓)	NA
PPAR α	↔	NA
PPAR γ	↔	↓
SF-1	↓	NA
Insl-3	↓ (↓)	NA (↓)
Dhh	↔	NA
Ptc-1	↔	NA

(↓) Downregulation/decreased expression in the current study; (↔) no significant effect of DEHP exposure in the current study; NA, not assessed in the current study; (↓) downregulation observed with DBP in other studies; (↑) upregulation observed with DBP in other studies.

that these nuclear receptors are involved in the testosterone reducing effect of phthalates.

Fig. 7 shows a schematic overview of the effects of DEHP on rat fetal testes that were observed in the present study as well as effects of DBP described in previous studies. It is indicated which genes are regulated by SF-1 and PPARs. As it is presently unclear which of the PPAR subtypes may be involved, we will here refer to “PPARs” in general instead of naming each PPAR subtype. The first step in testicular steroidogenesis is either uptake of cholesterol from blood or de novo cholesterol synthesis within the Leydig cell. Cholesterol uptake via SR-B1 is regulated by PPARs in the liver and macrophages (Chinetti et al., 2000; Malerod et al., 2003), and likely also in Leydig cells. PBR and StAR cooperate in the transport of cholesterol into the mitochondria, where P450scc converts cholesterol to pregnenolone. The remaining enzymes in the pathway (3 β -HSD, P450c17 and 17 β -hydroxysteroid reductase) are all located in the cytosol. SR-B1, StAR, P450scc, 3 β -HSD and P450c17 are regulated by SF-1, and PBR is regulated by PPARs (Val et al., 2003; Gazouli et al., 2002). Reduced steroid synthesis could thus be due to PPAR activation by DEHP, leading to downregulation of SR-B1 and PBR, or due to effects on SF-1 leading to reduced expression of SR-B1, StAR, P450scc, 3 β -HSD and P450c17 (Fig. 7).

The observed reduction in fetal testicular PBR mRNA expression corresponds with the observed reduction of PBR immunostaining in Leydig cells. PBR mRNA

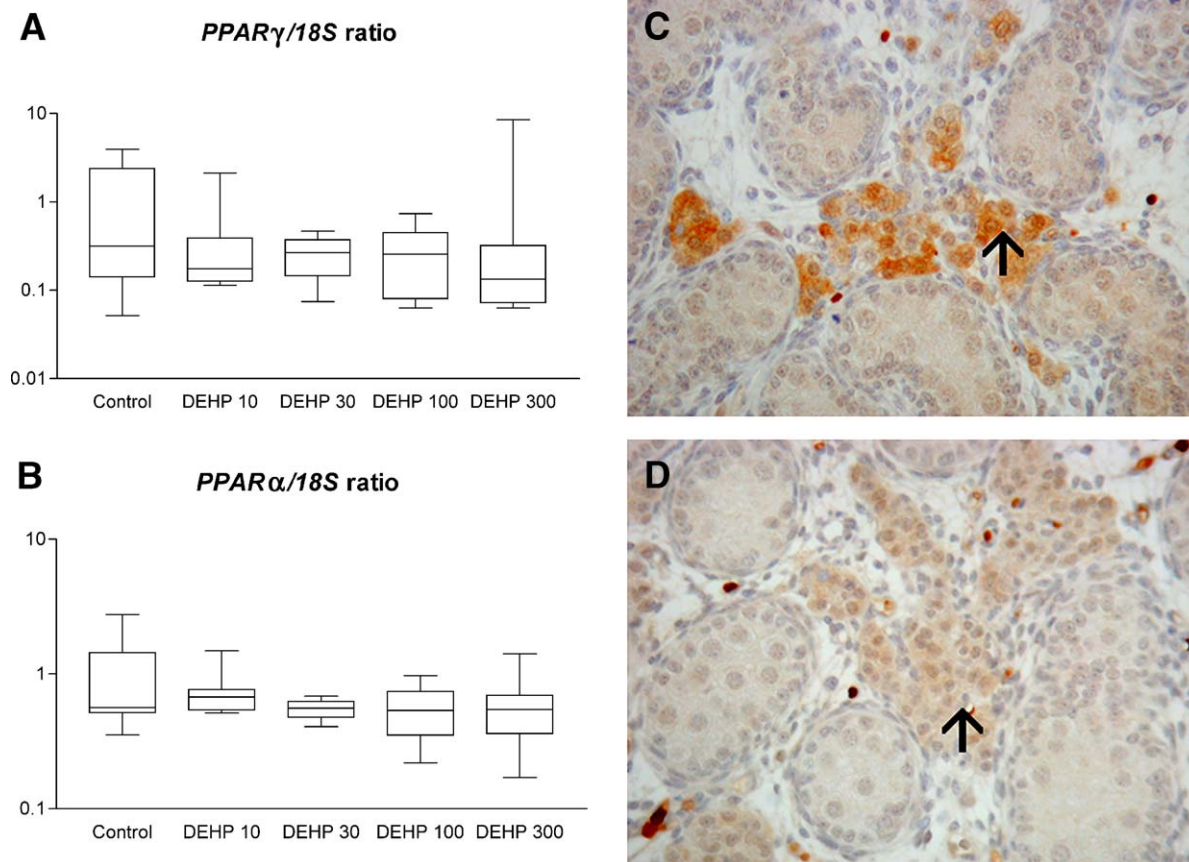


Fig. 6. Gene and protein expression of PPARs at GD 21 in testes of male rat fetuses exposed to 0, 10, 30, 100, or 300 mg DEHP/kg bw/day from GD 7 to GD 21. (A and B) mRNA levels of PPAR γ and PPAR α normalized to the levels of 18S (mean \pm S.E.M.). (C and D) The intensity of immunostaining in Leydig cells for PPAR γ is reduced in DEHP-exposed animals (D) compared to controls (C).

expression was also reduced in testes of adult mice exposed to DEHP (Gazouli et al., 2002), while reduced PBR mRNA expression in fetal testes has not been described previously for any phthalate. In contrast, Lehmann et al. (2004) found increased testicular PBR mRNA expression and reduced PBR immunostaining of Leydig cells in DBP-exposed rats at GD 19. The difference in the age of the fetus at the time of examination may explain the discrepancy between the gene expression results of the two studies.

As mentioned above, the nuclear receptor SF-1, which was downregulated in the current study, is involved in the regulation of several steroidogenic factors. Also the *Insl-3* gene contains binding sites for SF-1, indicating a role for SF-1 in the regulation of *Insl-3* expression (Koskimies et al., 2002). The observed reduction in *Insl-3* expression may thus be caused by an effect of DEHP on SF-1 activity. Testicular downregulation of *Insl-3* expression was also described in a study on 18-day-old fetal rats exposed to 750 mg DEHP/kg bw/day

(Wilson et al., 2004). In a study on male rats exposed postnatally (PND 5–14) to DBP, an increased expression of testicular SF-1 protein was observed on PND 20, supporting an involvement of SF-1 in the mechanism behind phthalate effects (Kim et al., 2004).

No ligands for the orphan receptor SF-1 have been described, but an interaction between phthalate monoesters and the nuclear receptor SF-1 may be speculated. However, Thompson et al. (2004) found no effect of monobutyl phthalate (MBP) on SF-1 regulated transcription of *StAR*, *SR-B1* or *P450c17* reporter constructs in a reporter gene assay. As no other phthalate metabolites were examined in that study, it is possible that DEHP metabolites and other metabolites of DBP than MBP are able to bind and activate SF-1.

In the current study, DEHP clearly reduced the immunohistochemical expression of PPAR γ protein in fetal rat testes. PPAR γ mRNA levels were not reduced as evidently as PPAR γ protein expression in Leydig cells, although most testes had very low PPAR γ transcript

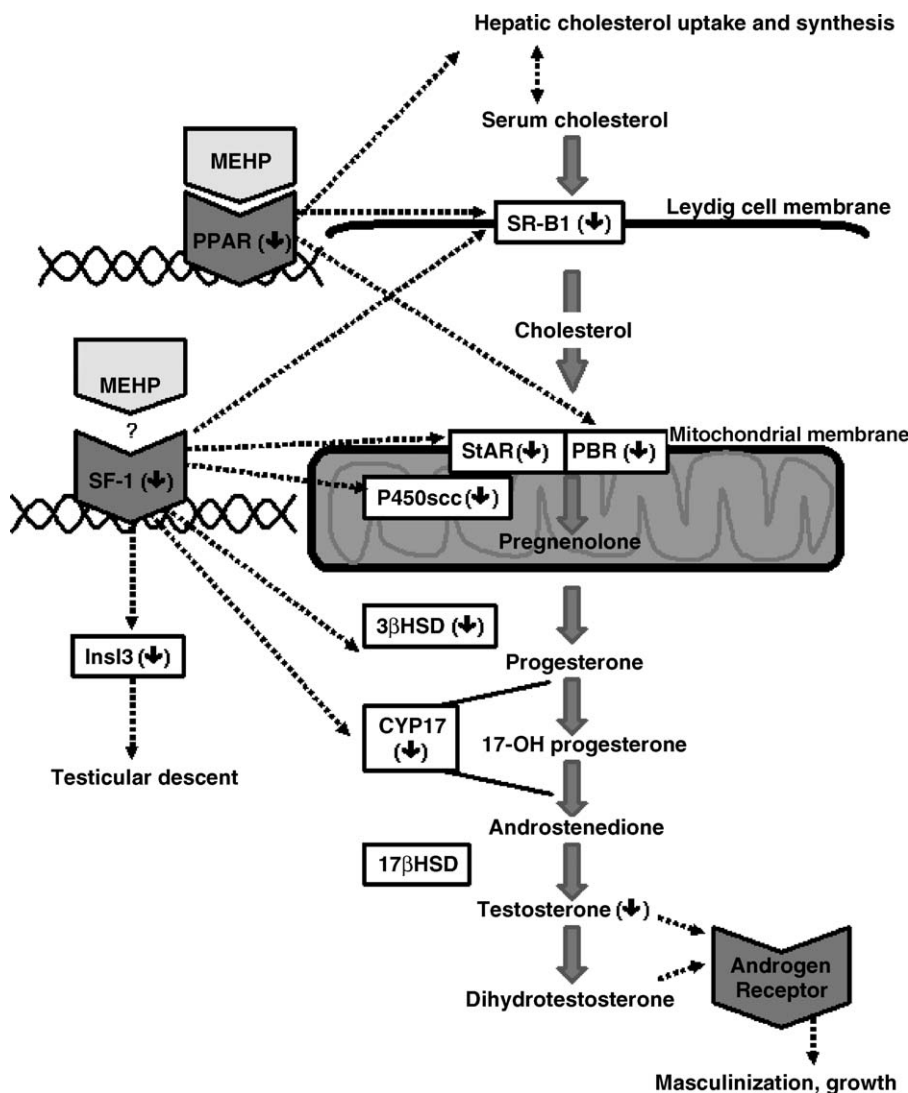


Fig. 7. Illustration of how phthalates may reduce testicular testosterone production and testicular descent via interaction with PPAR or SF-1. The uptake of cholesterol and conversion to testosterone involves numerous receptors and enzymes. (↓) Indicates factors (protein and/or mRNA) which are downregulated in fetal testes by DEHP in the current study or by DBP in other studies. The nuclear receptors PPAR and SF-1 regulate the expression of some of these factors as indicated by the dotted arrows. SR-B1, StAR, P450scc, 3β-HSD and P450c17 are regulated by SF-1, and PBR and possibly SR-B1 are regulated by PPARs. The low testosterone production in DEHP-exposed testes may thus be due to PPAR activation by phthalates, leading to downregulation of SR-B1 and PBR, or due to effects on SF-1 leading to reduced expression of SR-B1, StAR, P450scc, 3β-HSD and P450c17. As indicated, SF-1 also regulates InsI-3, which is associated with DEHP-induced cryptorchidism. MEHP, metabolite of DEHP, is known to bind and activate PPARs, but it is unclear whether MEHP interacts with SF-1.

levels compared to controls. This could be due to temporal differences in protein and gene expression and/or to the presence of PPAR γ expression in other cell types than Leydig cells (blood, macrophages). The observed reduction of PPAR γ protein levels in vivo by DEHP corresponds with the observation in vitro that PPAR γ activators downregulate PPAR γ protein and mRNA levels by autoregulation (Camp et al., 1999). It remains to be examined whether this low expression of PPAR γ upon

activation results in decreased or increased PPAR γ activity overall.

No clear effects on PPAR α mRNA expression were observed in fetal testes in the current study. In agreement with this, Ward et al. (1998) proposed that the testicular effects of phthalates are at least partly PPAR α independent, as DEHP-exposed PPAR α knockout mice and wild-type mice exhibited similar testis toxicity. Another study, however, showed reduced testosterone

levels in DEHP-exposed wild-type mice, but not in PPAR α knockout mice, indicating a role for PPAR α in effects on testosterone (Gazouli et al., 2002). Also the specific PPAR α agonist WY14,643, which does not activate PPAR γ or PPAR δ/β , was shown to suppress testosterone in vitro and in vivo (Gazouli et al., 2002). Although it is not clear which PPAR subtype may be involved in the testicular effects of phthalates, it is conceivable that PPAR α and PPAR γ (and possibly PPAR δ/β) share similar patterns of effects, and agonists to either of the subtypes may affect testosterone production.

The observed effect on SF-1 and PPAR γ expression on testes from GD 21 fetuses is difficult to relate causally to the reduction of testosterone production, as testosterone effects begin already at GD 17 (Parks et al., 2000). Any primary effect on nuclear receptor expression should thus preferably be investigated at or before GD 17, although the most sensitive time for detection of effects on testosterone is around GD 21 in our rat strain (Borch et al., 2006). Causal relations may be studied by investigation of rapid effects of phthalate treatment. In a study on acute DBP dosing of young rats, effects on PPAR regulated genes were observed already after 3–6 h (Kobayashi et al., 2003). Rapid effects on steroidogenic factors in testes of fetal rats exposed to DBP at GD 19 were studied by Thompson et al. (2005). They described an early drop in testicular testosterone levels within 1 h after maternal dosing. Six hours after treatment, gene expression of StAR, SR-B1, P450 scc and P450 $c17$ were reduced and followed by a further drop in testosterone levels (Thompson et al., 2005). The rapid reduction in testosterone levels indicates direct inhibition of enzymes involved in steroidogenesis, as the effect occurs before measured changes in gene expression of steroidogenic factors. The later drop in testosterone levels, however, may be related to regulation of steroidogenic enzymes by nuclear receptor such as PPARs and/or SF-1. Alternatively, the observed changes in PPAR γ and SF-1 expression may be secondary to a direct effect of DEHP on steroidogenic enzyme activity.

Reduced cholesterol availability may contribute to the reduced testosterone production. PPAR agonists increase the flux of cholesterol from extrahepatic tissues to the liver, as reviewed by Xie et al. (2002). Corresponding to this, reduced serum cholesterol was observed in adult male rats dosed with 125–1000 mg/kg bw/day of DEHP for 9 weeks (Daugaard et al., 2000). However, no change in plasma cholesterol was seen in fetuses exposed to DEHP doses up to 300 mg/kg bw/day in this study.

The human relevance of phthalate effects observed in rats has been debated, as hepatic PPAR α -mediated

effects are not considered relevant to humans (Doull et al., 1999). However, PPAR-mediated effects of phthalates in the testes may be highly relevant to humans. Indeed, the PPAR γ activating thiazolidinedione drugs rosiglitazone and troglitazone were found to reduce testosterone levels in normal men (Vierhapper et al., 2003), and are known to reduce the hyperandrogenism seen in women with Polycystic Ovary Syndrome (Bloomgarden et al., 2001). Human testes do express PPAR α in Leydig cells and spermatocytes, but human testicular expression of PPAR γ has not been adequately investigated (reviewed by Corton and Lapinskas, 2005).

In conclusion, this study revealed for the first time that gestational exposure to DEHP reduces nuclear receptors SF-1 mRNA and PPAR γ protein levels in fetal rat testes. Furthermore, the expression of several steroidogenic genes (SR-B1, StAR, PBR and P450 scc) was reduced by DEHP as previously shown for DBP, indicating a similar mechanism of action for DEHP and DBP. The inhibition of steroidogenesis leads to low fetal testosterone levels, likely causing adverse reproductive effects later in life. Additionally, reduced testicular expression of the cryptorchidism-associated *Insl-3* gene was observed. It is conceivable that the observed effects of DEHP on the expression of nuclear receptors SF-1 and PPAR γ are involved in the downregulation of steroidogenic factors and testosterone levels and thereby underlie the disturbed development of the male reproductive system.

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Diisobutyl phthalate has comparable anti-androgenic effects to di-*n*-butyl phthalate in fetal rat testis

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Abstract

Phthalates are widely used as plasticizers in various consumer products and building materials. Some of the phthalates are known to interfere with male reproductive development in rats, and di-*n*-butyl phthalate (DBP), diethylhexyl phthalate (DEHP) and butyl benzyl phthalate (BBP) were recently banned for use in toys in the EU mainly due to their reproductive toxicity. Diisobutyl phthalate (DiBP) has similar structural and application properties as DBP, and is being used as a substitute for DBP. However, knowledge on male reproductive effects of DiBP in experimental animals is lacking.

Methods: In the current study, four groups of pregnant Wistar rats were exposed to either 0 mg/kg bw/day or 600 mg/kg bw/day of DiBP from gestation day (GD) 7 to either GD 19 or GD 20/21. Male offspring was examined at GD 19 or GD 20/21 for effects on testicular testosterone production and testicular histopathology. Changes in anogenital distance (AGD) were evaluated as an indication of feminisation of males.

Results: Anogenital distance was statistically significantly reduced at GD 20/21 together with reductions in testicular testosterone production and testicular testosterone content. Histopathological effects (Leydig cell hyperplasia, Sertoli cell vacuolisation, central location of gonocytes and presence of multinuclear gonocytes) known for DBP and DEHP were observed in testes of DiBP-exposed animals at GD 20/21. Additionally, immunohistochemical expression of P450scc and StAR proteins in Leydig cells was reduced by DiBP. At GD 19, these effects on anogenital distance, testosterone levels and histopathology were less prominent.

Conclusion: In this study, GD 20/21 rather than GD 19 appears to be the optimal time for investigating changes in anogenital distance, testosterone levels, and testicular histopathology. DiBP has similar testicular and developmental effects as DBP and DEHP, and although more developmental and especially postnatal studies are needed to clearly identify the reproductive effects of DiBP, this study indicates a reason for concern about the use of DiBP as a substitute for DBP.

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Keywords: Phthalic acid ester; Diisobutyl phthalate; Prenatal; Steroidogenesis; Testis; Rats

1. Introduction

The observed increasing incidences of testicular cancer, hypospadias, cryptorchidism and reduced semen quality in recent years have been suggested to be caused

by endocrine disrupting environmental compounds with adverse effects on male reproduction (Skakkebaek et al., 2001). Among the chemicals suspected of causing these effects in humans are the phthalates, which are abundantly used in plastics, paints and other materials. Results from a recently published human study indicate associations between maternal phthalate exposure and reproductive development in their infant sons (Swan et al., 2005). Some of the most commonly used phtha-

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lates (dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP) and diisononyl phthalate (DiNP)) are known to induce adverse effects in the reproductive system of male rats after prenatal exposure (Barlow and Foster, 2003; Gray et al., 2000). Some of these effects are likely caused by a reduction in testicular testosterone production during late gestation (Barlow et al., 2003; Borch et al., 2004b; Parks et al., 2000).

Diisobutyl phthalate (DiBP) has not previously been examined in fetal rats, though it is widely used and suspected of having similar reproductive effects as the well-studied DBP. Due to its similarities to DBP, DiBP can be used as a substitute for DBP, which is presently used in PVC, inks, paints, adhesives and cosmetics (European Council for Plasticisers and Intermediates website, www.phthalates.com). Around 100 tons of DiBP is used in Denmark per year mainly as softeners in plastic, rubber, paint and glue, which accounts for 95% of the use. In the US, the monoester metabolite of DiBP, mono-isobutyl phthalate (MiBP), was found in women's urine in levels ranging from 0.7 ng/ml (25th percentile) to 5.1 ng/ml (75th percentile) (Swan et al., 2005). The exposure level of children to DiBP is unknown, but an estimate of the total exposure to DBP in Danish children aged 1–6 years is 400 µg/kg bw/day. This age group is estimated to be the group with the highest exposure level of DBP (Müller et al., 2003).

Since 1999 the six phthalates DEHP, DBP, BBP, DiNP, diisodecyl phthalate (DiDP) and di-*n*-octyl phthalate (DnOP) have been temporarily banned in the EU in the manufacture of toys and childcare articles for children under the age of three because of their carcinogenic, mutagenic and reprotoxic effects. Recently, DBP has along with DEHP and BBP been banned for use in toys irrespective of age categories due to their reproductive toxicity. DiNP, DiDP, and DnOP were concurrently banned in toys that can be put in the mouth by children.

The DiBP metabolite mono-isobutyl phthalate is found in human blood, saliva and urine (Silva et al., 2005). In a recent study, maternal urinary levels of MiBP (among other phthalate monesters) were associated with a short anogenital distance (AGD) in their infant boys (Swan et al., 2005). Anogenital distance is a commonly used measure of demasculinization of rats exposed to anti-androgenic chemicals.

In the current study, AGD, testicular testosterone levels, and testicular histopathology were evaluated in male rat fetuses exposed to DiBP. The effects were examined at gestation day (GD) 19 and GD 20/21 in order to determine the optimal time for detection of effects on fetal testosterone production.

2. Materials and methods

2.1. Test compounds

Diisobutyl phthalate (DiBP), CAS no. 84-69-5, purity 99%, Acros Organics, Geel, Belgium.

2.2. Animals and dosing

Twenty-four time-mated Wistar rats (HanTac:WH, Taconic M&B, Denmark, bodyweight approximately 210 g) were supplied at Day 3 of pregnancy. The day following mating was designated GD 1. The dams were randomized into four groups of eight with similar bodyweight distributions and housed in pairs under standard conditions. Semi-transparent plastic cages with Tapvei aspen bedding were situated in an animal room with controlled environmental conditions (12 h light–dark cycles with light starting at 9 p.m., light intensity 500 lx, temperature $21 \pm 2^\circ\text{C}$, humidity $50\% \pm 5\%$, ventilation 8 air changes per hour). Food (Altromin Standard diet 1324) and tap water were provided ad libitum. Dams were dosed daily by gavage from GD 7 to the day of autopsy with either vehicle (corn oil) or 600 mg/kg bw/day of DiBP. Animals were inspected for general toxicity twice daily. A control group and a dosed group were scheduled for autopsy at GD 19 and a control group and a dosed group for autopsy at GD 21.

Dams were delivered to the animal unit in four blocks mated on consecutive days and autopsied on consecutive days. As two of these blocks were mixed up on arrival, some dams were sacrificed 1 day earlier in pregnancy. Therefore, one fourth of the fetuses autopsied at “GD 21” were only 20 days old, and consequently named GD 20/21. As not all eight mated animals per group were pregnant, $n = 6$ litters per group on GD 19 and $n = 5$ litters in the control group and 6 litters in the DiBP group on GD 20/21.

At the day of autopsy, dams were anesthetized in CO_2/O_2 and decapitated, and fetuses were removed. AGD was measured in all fetuses using a dissecting scope with an ocular reticle. The measurements were performed blinded with respect to treatment group by a skilled technician who has many years of experience in measuring AGD on PND1 pups. Fetuses were decapitated and testes were removed and sampled for histopathology, measurement of testosterone production *ex vivo*, or measurement of testosterone content.

One or two testes per litter were placed in Bouin's fixative, and one or two testes per litter were placed in neutral buffered formaldehyde for histopathology and immunohistochemistry. One testis per litter was placed in 0.5 ml ice-cold Dulbecco's Modified Eagle Medium/F12 with 15 mM HEPES, 365 mg/l L-glutamine plus 0.1% bovine serum albumin and 0.1 g/l gentamicin. These testes were incubated at 37°C for 5 h, were then placed on ice, centrifuged at $4000 \times g$ for 10 min, and the supernatant was collected and stored at -80°C until analysis of testosterone content. Testosterone in supernatants was measured without further extraction.

One testis per litter was placed in an empty tube and immediately frozen in liquid nitrogen and stored at -80°C . Steroid

hormones were analyzed in testes after extraction with diethyl ether. Testes were placed in vials containing 100 µl water, extracted with 0.5 ml diethyl ether, and the procedure was repeated. Following evaporation, vials were added 100 diluent 1 (zero calibrator from Perkin–Elmer Life Sciences, Turku, Finland) and samples were vortexed and incubated for 10 min at 45 °C before analysis.

2.3. Analyses of testosterone levels

Testosterone content in incubation media and testes was analyzed with a Delfia time-resolved fluoroimmunoassay (Perkin–Elmer, Wallac Oy, Turku, Finland). Samples and standards were measured in duplicates and the CV% were always <20% (mean CV of 6.9%).

2.4. Histopathology and immunohistochemistry

One section per Bouin's or formaldehyde fixed testis was stained with H&E for histopathological evaluation and was evaluated by an observer blinded with respect to treatment groups. Immunohistochemistry was performed on one section per Bouin's-fixed testis. Following microwave pretreatment for 2 × 5 min in either citrate or TEG buffer, sections were blocked for endogenous peroxidase activity in 3% H₂O₂ in PBS, and blocked in 1% bovine serum albumin in PBS. Sections were incubated over night at 4 °C with the following rabbit polyclonal antibodies: steroidogenic acute regulated protein (StAR) antibody (PA1-560, Affinity Bioreagents, Golden, CO, 1:1000), P450side chain cleavage (P450_{scc}) antibody (AB 1244, Chemicon, Temecula, CA, 1:50,000). Sections were then incubated for 30 min with secondary antibody (Anti-rabbit EnVision+, DAKO, Glostrup, Denmark), stained in diaminobenzidine (DAB+ Substrate Chromogen System, DAKO, Glostrup, Denmark), and counterstained in Meyer's hematoxylin. Intensity of staining was scored as "normal" or "reduced" staining by an observer blinded with respect to treatment groups.

2.5. Statistics

Non-processed, logarithmically transformed and square root transformed data were examined for normal distribution and homogeneity of variance. Normally distributed data were analyzed by ANOVA using single animal data. For datasets including more than one animal per litter, the litter was the statistical unit and included in the analysis of variance as an independent, random factor nested within treatment group. AGD data were analyzed for males and females separately, and with cubic root of bodyweight as a covariate. A Fisher's Exact Test was used to analyze histological changes in the testes at GD 19 and GD 20/21. SAS statistical software was used for the analyses (SAS Institute Inc, Cary, NC, USA). Asterisks indicate a statistically significant difference compared to controls * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3. Results

As previous studies have shown effects of DEHP, DBP and DiNP on testosterone production, anogenital distance and testicular histopathology in male fetal rats, these endpoints were examined at two different gestational ages in male rats exposed to 600 mg/kg bw/day of DiBP.

At GD 19 and GD 20/21, testicular testosterone content and testicular testosterone production ex vivo were reduced in the DiBP group compared to controls (Fig. 1). These effects were statistically significant at GD 20/21 ($p < 0.0001$ and $p = 0.0003$ for testicular testosterone content and testosterone production, respectively) but not at GD 19 ($p = 0.08$ and $p = 0.33$). As mentioned, some fetuses in the GD 20/21 group were only 20 days old, but statistical comparison of data from the two age groups showed no relevant differences, why all fetuses were included in the GD 20/21 group.

Corresponding to the reduced testosterone levels, AGD was significantly reduced in DiBP dosed males compared to controls at GD 19 and GD 20/21 ($p = 0.009$ and $p = 0.009$), indicating reduced masculinization (Fig. 2). AGD was increased in female DiBP-exposed

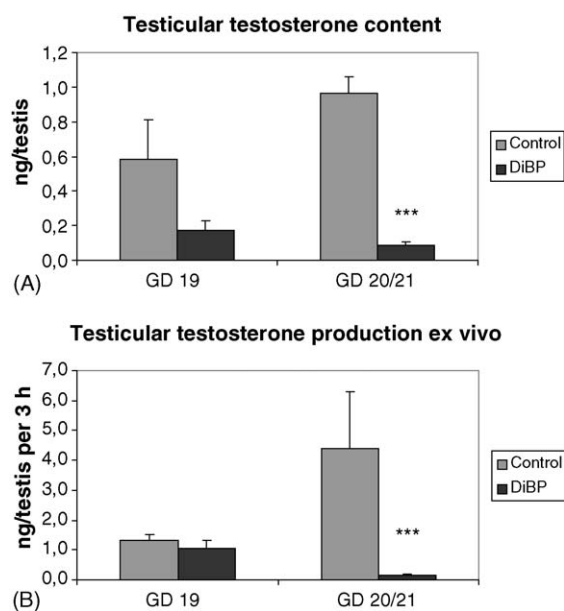


Fig. 1. Testosterone levels at GD 19 and GD 20/21 in male rat fetuses exposed to 0 mg DiBP/kg bw/day or 600 mg DiBP/kg bw/day from GD 7 to GD 19 or GD 20/21. (A) Testicular testosterone content ($n = 5-6$); (B) testicular testosterone production ex vivo ($n = 5-6$). Figures show mean \pm S.E.M. Asterisks indicate a statistically significant difference compared to controls *** $p < 0.001$.

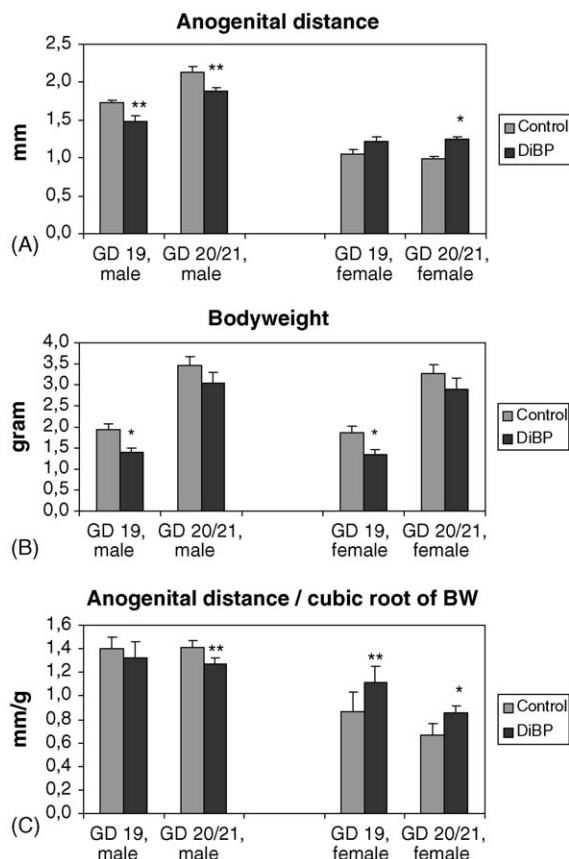


Fig. 2. Anogenital distance (A), bodyweights (B), and anogenital distance/cubic root of bodyweight (C), at GD 19 and GD 20/21 in male and female rat fetuses exposed to 0 mg DiBP/kg bw/day or 600 mg DiBP/kg bw/day from GD 7 to GD 19 or GD 20/21. Figures show mean \pm S.E.M. Asterisks indicate a statistically significant difference compared to controls * $p < 0.05$, ** $p < 0.01$.

fetuses compared to controls. This was statistically significant at GD 20/21 ($p = 0.02$), but not at GD 19 ($p = 0.057$). We have not previously measured AGD on animals taken by Caesarian section, and therefore have no historical control values for fetuses. However, the historical control data on AGD in PND1 pups show the same difference between the sexes as we observed in this study, i.e. males have an AGD that is approximately twice as long as that of the females. Bodyweight was also lower in the DiBP-exposed males and females (statistically significant at GD 19 but not GD 20/21) and therefore the cubic root of bodyweight was included as a covariate in the statistical analysis in order to take account for the influence of body size on AGD. When analyzing AGD divided by the cubic root of bodyweight, as shown in Fig. 2(C), we found that DiBP dosed males had significantly lower values than controls

Table 1

Testicular histopathology in male rat fetuses exposed to 0 mg DiBP/kg bw/day or 600 mg DiBP/kg bw/day from GD 7 to GD 19 or GD 20/21

	GD 19		GD 20/21	
	Control	DiBP	Control	DiBP
Clustering of small Leydig cells	2/13	9/9***	0/10	13/15***
Sertoli cells vacuolisation	0/13	1/9	0/10	14/16***
Central localization of genocytes	0/13	2/9	0/10	14/16***
Multinuclear genocytes	1/13	0/9	1/10	10/16*
Reduced intensity of StAR staining in Leydig cells	0/8	4/5**	0/4	5/5**
Reduced intensity of P450scc staining in Leydig cells	0/8	2/5	0/4	7/7**

Numbers are single animal data on one testis from each of one to three males per litter. There were 5 litters per group on GD 19 and 5 control litters and 6 DiBP litters on GD 20/21.

* $p < 0.05$, statistically significant difference compared to controls.

** $p < 0.01$, statistically significant difference compared to controls.

*** $p < 0.001$, statistically significant difference compared to controls.

on GD 20/21 ($p = 0.008$) but not on GD 19, while the dosed females had significantly higher values than control females on both GD 19 and GD 20/21 ($p = 0.005$ and $p = 0.032$). There was no significant effect of dosing on maternal weight gain during pregnancy, and no difference on litter size, fetal viability, and number of resorptions (data not shown).

DiBP-exposed animals had the histopathological characteristics also seen with other phthalates at GD 20/21, as listed in Table 1. The number of gonocytes appeared to be increased, and they were located centrally in the seminiferous tubules due to vacuolization of Sertoli cell cytoplasm. Leydig cells were found in clusters and had small cytoplasm and small irregular nuclei (Fig. 3). On GD 20/21, these effects were seen in almost all examined testes, when examining one section per testis. On GD 19, the effect of DiBP was mainly on Leydig cell size and number, while Sertoli cell and gonocyte effects were only observed in one or two of the examined testes (Table 1).

StAR and P450scc staining intensity was reduced in Leydig cells of DiBP-exposed animals compared to controls (Table 1 and Fig. 4). This indicates reduced expression of these two proteins and thereby a reduced capacity of the steroidogenic pathway leading to the observed reduction in testosterone levels.

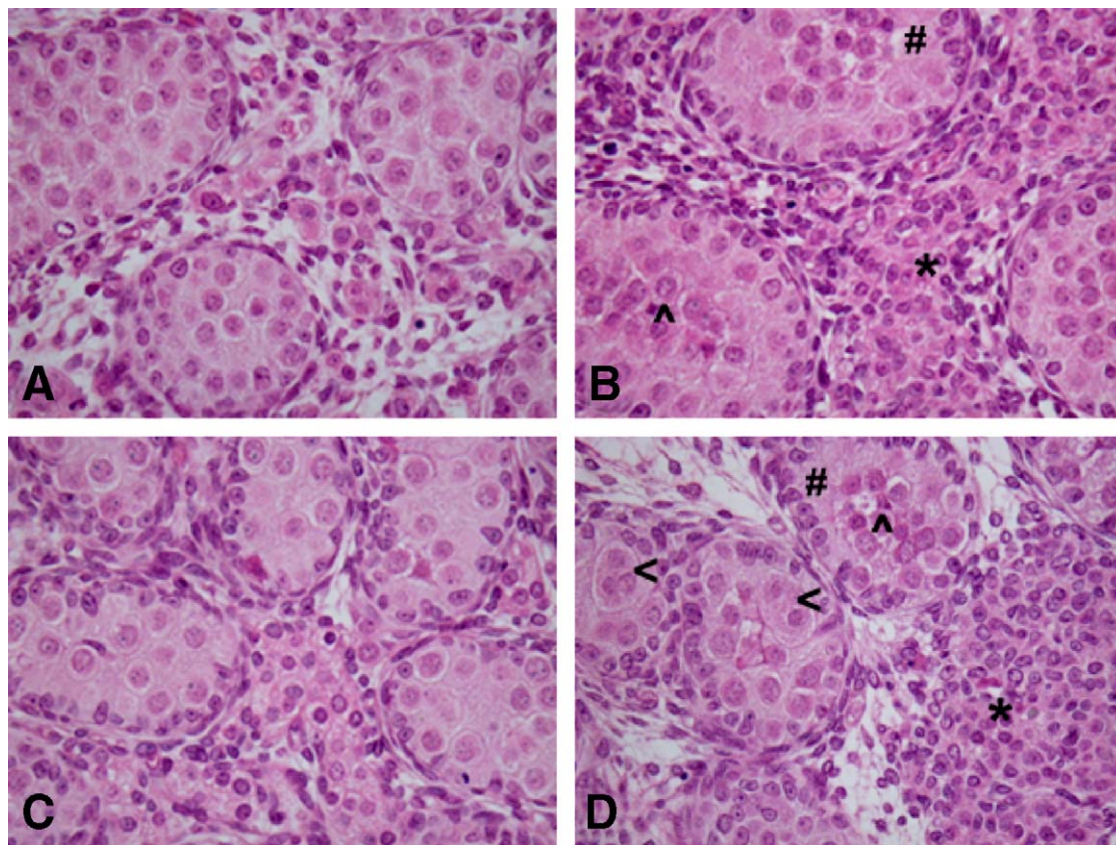


Fig. 3. Testicular histopathology at GD 19 (A + B), and GD 20/21 (C + D), in male rat fetuses exposed to vehicle (A + C), or 600 mg DiBP/kg bw/day (B + D), from GD 7 to GD 20/21. DiBP-exposed animals had a larger number of gonocytes, which were located centrally in the seminiferous tubules (\wedge) due to vacuolization of Sertoli cell cytoplasm ($\#$). Leydig cells ($*$) of DiBP-exposed animals were found in clusters and had small cytoplasm and small irregular nuclei. ($<$) Multinucleated gonocytes. Original magnification 40 \times .

4. Discussion

The current study shows that DiBP has similar effects as DEHP, DBP and DiNP on rat fetal testicular testosterone production and testicular histopathology following exposure in utero. The proteins StAR and P450scc involved in steroid synthesis in testes were also reduced by DiBP as shown for other phthalates (Barlow et al., 2003; Borch et al., submitted).

Due to the increasing knowledge on adverse reproductive effects of some of the most widely used phthalates, the industrial use of less well investigated phthalates is increasing. DiBP is widely used, but only few studies have examined its reproductive effects. Reduced testis weight and increased testicular testosterone content was observed in 5-week-old rats exposed to 2% DiBP in their diet (Oishi and Hiraga, 1980c). On the other hand, a study in 5-week-old mice showed that 2% dietary MiBP along with MEHP and MBP

reduced testicular testosterone levels, whereas absolute testis weights was not affected (Oishi and Hiraga, 1980b). In contrast to their results with monoesters, the same authors did not observe any effect of DiBP, DEHP and DBP on absolute testes weights in mice (Oishi and Hiraga, 1980a). Testicular atrophy was observed in a study on prepubertal rats exposed to 800 mg/kg bw/day MiBP for 6 days (Foster et al., 1981). A developmental toxicity study by the European Council for Plasticisers and Intermediates (ECPI) showed reduced maternal bodyweight gain and reduced fetal weight in rats exposed to 942 mg/kg bw/day of DiBP from GD 6 to GD 20, while no embryo-/fetotoxicity was seen at 363 mg/kg bw/day or 88 mg/kg bw/day (European Council for Plasticisers and Intermediates website, www.phthalates.com). However, that study did not examine the early endocrine effects or late reproductive effects in male offspring. Studies with DBP have shown that male reproductive development is affected

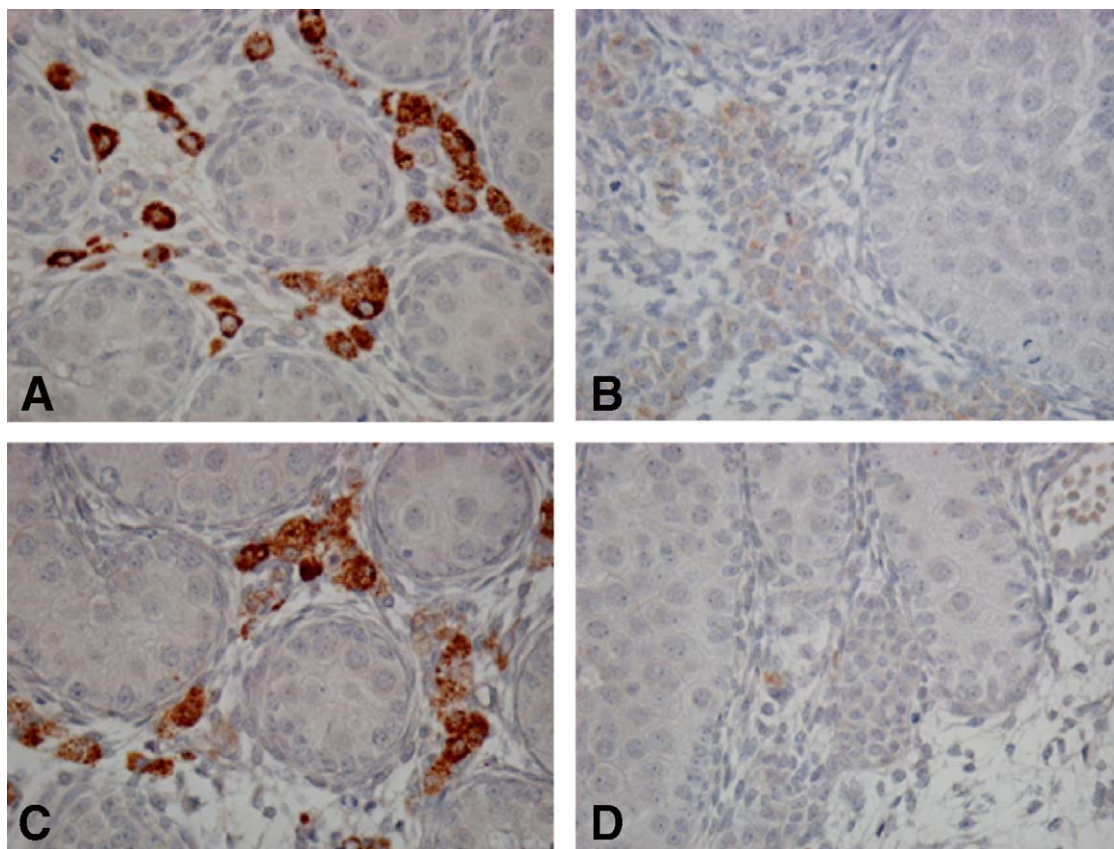


Fig. 4. Immunohistochemical staining of testes from 20/21-day-old male rat fetuses exposed to 0 mg DiBP/kg bw/day or 600 mg DiBP/kg bw/day from GD 7 to GD 20/21. The intensity of staining in Leydig cells for StAR (A + B), and P450scc (C + D), is clearly reduced in DiBP-exposed animals (B, D) compared to controls (A, C). Original magnification 40 \times .

at much lower doses than those inducing teratogenicity (Lehmann et al., 2004).

DiBP appears to disrupt fetal testicular development as potently as the well-known testicular toxicants DEHP and DBP, and more potently than DiNP. In Table 2, the effects of DiBP on testicular testosterone production and testicular testosterone levels in the current study are compared to similar analyses described in the literature for other phthalates. It should be noted that measurements in the listed studies were made on other days of gestation and with other phthalate doses. When comparing to our own previously published data, it is clear that the effects observed with 600 mg/kg of DiBP at GD 20/21 in the current study are at least as marked as effects of 750 mg/kg and 300 mg/kg of DEHP and 750 mg/kg of DiNP at the same age (Table 2). However, testosterone values generally show some variation in our studies, as mean control values can vary up to two-fold between studies (Borch et al., 2004b). Also the severity and incidence of histopathological effects of DiBP is comparable to that seen with DEHP in our previous studies (Borch et

al., 2005) and is higher than the incidence of histopathological effects observed with DiNP (Borch et al., 2004a).

None of the studies by other groups have been performed at GD 20/21, and direct comparison to those studies is therefore difficult. In this study, we found more marked effects at GD 20/21 compared to GD 19. Similarly, Table 2 shows that the effect of DBP and DEHP was more marked at the later ages of gestation in the studies by Thompson et al. (2004) and Parks et al. (2000). The weak effect of DiBP at GD 19 in the current study may indicate a reduced sensitivity of our assay compared to studies by other groups.

AGD was reduced at GD 19 and GD 20/21 in males and increased in females exposed to DiBP. DiBP reduced bodyweights of male and female fetuses, but when analysing AGD data with bodyweight as a covariate, the impact of reduced body size was taken into account. Reduced male AGD measured in infancy is commonly observed following exposure to anti-androgenic chemicals and has also been described for other phthalates (Gray et al., 2000; Jarfelt et al., 2005). The observed

Table 2
Overview of studies on fetal testosterone levels in phthalate exposed rats

Test compound	Dose (mg/kg bw/day)	Examination day	Testicular testosterone production ex vivo, % of control (control = 100%)	Testicular testosterone content, % of control	Reference
BBP	1000	GD 18	13		Wilson et al. (2004)
BBP	500	GD 18	53	63	Hotchkiss et al. (2004)
DBP	1000	GD 18	10		Wilson et al. (2004)
DBP	500	GD 17		48	Thompson et al. (2004)
DBP	500	GD 18		19	Thompson et al. (2004)
DBP	500	GD 19		10	Thompson et al. (2004)
DEHP	1000	GD 18	47		Wilson et al. (2004)
DEHP	750	GD 17	44		Parks et al. (2000)
DEHP	750	GD 18	43		Parks et al. (2000)
DEHP	750	GD 20	11		Parks et al. (2000)
DEHP	750	GD 21	18	23	Borch et al. (2004b)
DEHP	300	GD 21	21	30	Borch et al. (2004b)
DiNP	750	GD 21	31	27	Borch et al. (2004b)
DiBP	600	GD 19	79 (NS)	30	Current study
DiBP	600	GD 20/21	4	9	Current study

It should be noted that different study designs, involving different strains of rats and dosing periods, were used (see relevant references). NS: no significant difference compared to controls.

increase in female AGD has not been described previously for any phthalate, but the anti-androgenic pesticide prochloraz has similarly been shown to increase female AGD (Laier et al., *in press*). We do not know the mechanisms behind this finding, but suspect that this is caused by a hormonal imbalance in the DiBP-exposed female fetuses. The reason why this effect has not been reported in other phthalate studies may be that the focus of perinatal studies has been on the anti-androgenic properties of the compounds, which are most marked in male offspring.

This study was designed to investigate whether the effects of DiBP were comparable to DEHP, DBP, and DINP on testosterone synthesis, AGD and histopathology and therefore only one relatively high dose of DiBP was used. In this study, no NOAEL can be provided for risk assessment purposes. Among other metabolites of phthalates, Swan et al. (2005) measured MiBP in the urine of women in the childbearing age, indicating that sensitive age groups are exposed to several phthalates including DiBP. With the current study, DiBP is recognized as yet an additional inhibitor of fetal steroidogenesis. In this study, GD 20/21 appears to be the optimal time for investigating changes in anogenital distance, testosterone levels, and testicular histopathology. Based on the current results, it appears likely that DiBP has similar reproductive effects to DBP later in life, such as testicular and epididymal atrophy or agenesis and reduced semen quality. DEHP, DBP and BBP were recently banned for use in toys in the EU due to their reproductive toxicity. The current study provides new evidence for a similar

reproductive toxicity of DiBP, and indicates a reason for concern about the use of DiBP as a substitute for DBP.

Conflict of interest statement

There are no conflicts of interest.

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Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz

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Abstract

The fungicide prochloraz has got multiple mechanisms of action that may influence the demasculinizing and reproductive toxic effects of the compound. In the present study, Wistar rats were dosed perinatally with prochloraz (50 and 150 mg/kg/day) from gestational day (GD) 7 to postnatal day (PND) 16. Caesarian sections were performed on selected dams at GD 21, while others were allowed to give birth to pups that were followed until PND 16. Prochloraz caused mild dysgenesis of the male external genitalia as well as reduced anogenital distance and retention of nipples in male pups. An increased anogenital distance indicated virilization of female pups. Effects on steroidogenesis in male fetuses became evident as decreased testicular and plasma levels of testosterone and increased levels of progesterone. Ex vivo synthesis of both steroid hormones was qualitatively similarly affected by prochloraz. Immunohistochemistry of fetal testes showed increased expression of 17 α -hydroxylase/17,20-lyase (P450c17) and a reduction in 17 β -hydroxysteroid dehydrogenase (type 10) expression, whereas no changes in expression of genes involved in testicular steroidogenesis were observed. Increased expression of P450c17 mRNA was observed in fetal male adrenals, and the androgen-regulated genes ornithine decarboxylase, prostatic binding protein C3 as well as insulin-like growth factor I mRNA were reduced in ventral prostates PND 16. These results indicate that reduced activity of P450c17 may be a primary cause of the disrupted fetal steroidogenesis and that an altered androgen metabolism may play a role as well. In vitro studies on human adrenocortical carcinoma cells supported the findings in vivo as reduced testosterone and increased progesterone levels were observed. Overall, these results together indicate that prochloraz acts directly on the fetal testis to inhibit steroidogenesis and that this effect is exhibited at protein, and not at genomic, level.

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Keywords: Prochloraz; Steroidogenesis; Antiandrogen; Rat; Leydig cell

Introduction

Increased incidence of human reproductive health effects in males such as malformed sex organs, poor semen quality and testicular cancer have been reported in many industrialized countries (Toppari et al., 2002). The first indication of a link to endocrine-active chemicals has recently been published, showing that feminization of sex organs in newborn boys was correlated to prenatal phthalate exposure (Swan et al., 2005).

Furthermore, previous epidemiological studies have indicated a causal connection between human exposure to pesticides and endocrine disrupting effects such as poor sperm quality (Swan et al., 2003) and increased incidence of cryptorchidism in sons of gardeners (Weidner et al., 1998).

An increasing amount of chemicals have been demonstrated to act as antiandrogens in experimental animals including the thoroughly studied pesticides linuron, procymidone, and vinclozolin together with diethylhexylphthalate (DEHP) and dibutylphthalate (DBP) (Gray et al., 1999a, 1999b, 2001; Thompson et al., 2005). Some of these antiandrogens have been banned and substituted with alternatives, but still many pesticides currently used in the industrialized world are

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suspected of having various endocrine disrupting effects as shown by in vitro screening (Andersen et al., 2002).

The pesticide prochloraz is currently used in a great part of the world within agriculture and horticulture due to its fungicidal effects. Prochloraz has been shown to act via multiple mechanisms of action in vitro as it antagonizes the androgen and the estrogen receptor, agonizes the arylhydrocarbon receptor and inhibits aromatase activity (Andersen et al., 2002; Long et al., 2003; Mason et al., 1987). Additionally, in vivo studies on male rats have demonstrated prochloraz to act as an antiandrogen in the Hershberger assay, in which the lowest tested dose of 50 mg/kg was causing reduced weights of reproductive organs, down-regulated mRNA levels of androgen-regulated genes in prostates and increased serum LH levels (Vinggaard et al., 2002).

Developmental toxic effects of prochloraz have been reported in two recent studies (Vinggaard et al., 2005; Noriega et al., 2005). Perinatal exposure to 30 mg/kg prochloraz caused delayed delivery of the pups and increased nipple retention and reduced weights of bulbourethral glands in newborn male pups (Vinggaard et al., 2005). Furthermore, feminized behavior of the adult males in this study was expressed as an increased activity level and an increased intake of sweetened water, indicating that exposure during gestation and lactation causes permanent adverse effects in adulthood. In agreement with previous results reported by Wilson et al. (2004), these effects were accompanied by a marked reduction of fetal testosterone production and increased progesterone production, and it was suggested that the feminization of the male offspring was at least partly due to this mechanism of action. In male rats exposed in utero for 5 days to 31.25, 62.5, 125, and 250 mg/kg prochloraz, developmental effects in male pups such as reduced anogenital distance, nipple retention, hypospadias, severe phallus clefting and exposure of the os penis were observed together with observations that vaginal morphology was induced in males (Noriega et al., 2005). It was suggested that higher doses of prochloraz produce a profile of effects that resemble the profile produced by lower doses of androgen receptor antagonists like flutamide and vinclozolin. Overall, prochloraz is able to feminize male rat offspring after perinatal exposure, but the detailed mechanisms of actions that are involved remain to be elucidated.

Steroidogenesis and production of testosterone during gestation are critical for function and development of the male reproductive organs. In male rat fetuses, the antiandrogenic profile of phthalates such as DBP is distinct from that of typical androgen receptor antagonists such as flutamide, but they still elicit relatively similar reproductive abnormalities such as hypospadias and absent or deformed seminal vesicles and ventral prostates (Mylchreest et al., 2000). However, DBP does not seem to block the androgen receptor to a major extent but rather interfere with the androgen synthesis resulting in diminished testosterone production. Examination by quantitative real-time RT-PCR of the expression of various genes involved in androgen biosynthesis and signaling has formed part of the studies on mechanisms of action lying behind the antiandrogenic effects caused by DBP (Shultz et al., 2001; Barlow et al., 2003). Down-regulation of mRNA expression of

scavenger receptor class B-1 (SRB1), steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage enzyme (P450_{scc}), 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and 17 α -hydroxylase/17,20-lyase (P450_{c17}) by DBP in fetal testes suggests that cholesterol uptake and transport and its further conversion are potential mechanisms for the decreased testosterone synthesis (Barlow et al., 2003; Lehmann et al., 2004; Thompson et al., 2004). Consequently, it is of great interest to investigate whether these mechanisms of action play a role in the steroidogenic effects of prochloraz as well.

The purpose of the present study was to perform a detailed investigation of the mechanisms of action responsible for the prochloraz-induced effects on fetal steroidogenesis in male rat pups. Critical genes and proteins involved directly or indirectly in steroidogenesis and/or Leydig cell function were analyzed in the testes and adrenals by quantitative real-time RT-PCR, and the same method was applied to look for changes in expression of androgen-regulated genes in prostates from male pups. These investigations were supplemented with immunohistochemistry of relevant proteins and with hormone analyses in order to elucidate the mechanisms responsible for the observed effects. Finally, hormone levels were measured in mouse Leydig cells and human adrenocortical cells in order to find a suitable in vitro model that qualitatively reflects the effects of prochloraz on fetal steroidogenesis in vivo.

Methods

Test compounds

Prochloraz 99.3% pure (CAS no. 67747-09-5; *N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]-1*H*-imidazole-1-carboxamide) from Institute of Organic Chemistry (Warsaw, Poland) was used for the in vivo studies, while prochloraz 99.4% pure from Riedel de H  en (Seelze, Germany) was used for in vitro studies. The test compound was dissolved in peanut oil (no. P-2144) from Sigma-Aldrich (St. Louis, USA) for in vivo studies.

Animals and exposure

Fifty-six young adult nulliparous Wistar rats (HanTac: WH, Taconic M and B, Ejby, Denmark) were supplied at day 3 of pregnancy. The animals were distributed, housed and handled as previously described (Vinggaard et al., 2005). At the day after arrival, i.e. gestational day (GD) 4, animals were weighed and assigned to 3 groups of 24, 16 and 16 rats respectively with similar weight distributions. The rats were gavaged with 0, 50 mg/kg or 150 mg/kg of prochloraz from gestational day 7 to postnatal day (PND) 16. In the 3 groups, 21, 16 and 14 rats, respectively, appeared to be pregnant.

Health status of dams and delivery

Time of birth and the health status of the dams were monitored as previously described by Vinggaard et al. (2005). Among the time-mated animals, 13, 8 and 6 dams from the control, 50 mg/kg and 150 mg/kg prochloraz group, respectively, gave birth. The pups were counted, sexed and checked for anomalies. Pups found dead were investigated macroscopically for alterations when possible. The expected day of delivery, GD 22, was designated PND 0 for the pups.

Caesarian sections GD 21

Eight dams were randomly selected from each group for Caesarian section at GD21. From the control, 50 mg/kg and 150 mg/kg prochloraz group, respectively, 6, 7 and 5 dams were pregnant. The dams were weighed and

decapitated after CO₂/O₂ anesthesia, uterus was taken out, and the number of live fetuses, resorptions and implantations was registered. Location in uterus, sex, nipples and any anomalies were recorded. Blood was taken from the trunk of all fetuses immediately after decapitation in heparin-coated vials for hormone analysis, and samples were pooled from each litter for both males and females. From each litter, one testis was taken for determination of testosterone (stored in –80 °C freezer until analysis) and one for histopathology (fixed in Bouin's fixative). Paired testes and adrenals were taken from litters with two or more males and stored in RNAlater (Qiagen, Crawly, UK) for gene expression analyses. When there were more than two males in a litter, one testis was fixed in formalin for histopathology and immunohistochemistry, while the other testis was analyzed for progesterone. In litters with additional males, testes were taken and ex vivo testosterone production was determined (processed immediately).

Anogenital distance and nipple retention

Anogenital distance (AGD) was measured in the offspring at birth using a stereomicroscope. The analysis of AGD was performed by dividing the AGD with the cubic root of the pup body weight.

On PND 13, the pups were examined for the presence of areolas/nipples, described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. The pups' body weights were measured at both time points.

Section of pups PND 16

Body weights of all male pups were recorded, and they underwent a thorough autopsy at PND 16. The following organs were excised and weighed: liver, kidneys, adrenals, testes, epididymides, seminal vesicles, ventral prostate, bulbourethral glands and levator ani/bulbocavernosus muscle. Additionally, the thyroids were excised but not weighed ($n = 5$ to 8 males per age group per dose group).

For each litter at PND 16, the right or left testes were alternately fixed in Bouin's fixative, while the other was frozen for analysis of testosterone levels. From litters with two or more males, paired testes, adrenals and liver were weighed and stored in RNAlater for gene expression analyses. When litters had three or more males, one testis was fixed in formalin, and one was frozen for analysis of progesterone levels. The thyroid, adrenals, epididymides, seminal vesicles and ventral prostate were fixed in formalin, and all fixed organs were embedded in paraffin and examined by light microscopy after staining with hematoxylin and eosin. Testes taken for determination of hormone levels were stored in –80 °C freezer until analysis.

In a supplementary in vivo study with similar animals, exposure period and other experimental conditions, 6, 7 and 8 pregnant Wistar rats, respectively, were exposed to 0, 50 and 100 mg/kg prochloraz. The ventral prostates from PND 16 males were weighed and stored in RNAlater for gene expression analyses.

Investigation of male external genitalia

The external genitalia were inspected at PND 16 in all males from all litters. The changes were scored on a scale from 1 to 4 in order to investigate whether male external genitals were feminized. The following criteria were used: Score 1: No effect. Normal genital tubercle, the urethral opening, is found at the tip of the genital tubercle, and the preputial skin is intact. In the perineal area, thick fur extends caudally from the base of the genital tubercle half the distance to the anus. A furless area circumscribes the anus. Score 2: Mild dysgenesis of the external genitalia: a small cavity on the inferior side of the genital tubercle and a minor cleft in the preputial opening are observed, estimated 0.5–1.4 on an arbitrary scale. The furless area around anus expands towards the base of the genital tubercle, but thick fur is still present at the base of the genital tubercle. Score 3: Moderate dysgenesis of the external genitalia: the preputial cleft is larger, estimated 1.5–2.4 on an arbitrary scale. The urethral opening is situated halfway down the inferior side of the genital tubercle (hypospadias). Partly furless or thin fur in the perineal area range from the base of the genital tubercle and caudally to the furless area circumscribing the anus. Score 4: Severe dysgenesis of the external genitalia: The preputial cleft is large, estimated 2.5–3.5 on an arbitrary scale. The urethral opening is situated more than halfway

from the tip to the base. At the base of the genital tubercle, a groove extending laterally is observed (similar to control females at PND 16). Perineal area is totally furless.

Immunohistochemistry of fetal testes

Immunohistochemical staining was performed on four to five 4 µm sections from each GD 21 testis kept in Bouin's fixative from the control group and the 150 mg/kg prochloraz group. Following microwave pretreatment for 2 × 5 min in either citrate or T-EG buffer, sections were blocked for endogenous peroxidase activity in 3% H₂O₂ in PBS and blocked in 1% bovine serum albumin in PBS. Sections were incubated overnight at 4 °C with rabbit polyclonal antibodies against StAR (PA1-560, Affinity Bioreagents, Golden, CO), P450scc (AB 1244, Chemicon, Temecula, CA), 17β-hydroxysteroid dehydrogenase (17β-HSD) type 10 (Biotrend Chemicals, Köln, Germany), 3β-HSD or p450c17 (both antibodies were kind gifts from Dr. I. Mason, Edinburgh, UK). Sections were then incubated for 30 min with anti-rabbit EnVision+ (DAKO, Glostrup, Denmark), stained in DAB+ (DAKO, Glostrup, Denmark) and counterstained in Meyer's hematoxylin. Negative controls were fetal control testis incubated with blocking serum instead of primary antibody.

Hormone analysis

Testosterone, progesterone, LH and thyroid hormones (T3, T4 and TSH) were analyzed in rat serum or testes at GD 21 and/or PND 16. Steroid hormones were extracted on IST Isolute SPE columns from the serum as previously described (Vinggaard et al., 2002; Birkhoj et al., 2004), and these hormones together with the thyroid hormones triiodothyronine (T3) and thyroxine (T4) were measured by time-resolved fluorescence using commercially available FIA kits (PerkinElmer Life Sciences, Turku, Finland). LH was analyzed at Turku University, Finland, using an immunofluorimetric assay as described by Haavisto et al. (1993). Rat TSH was determined by an enzyme immunoassay BioTrak from Amersham Biosciences (Uppsala, Sweden). Inhibin B was measured using an Inhibin B ELISA assay (Oxford Bio-Innovation, Oxford, UK) recognizing both the βB subunit and the α-subunit of inhibin B. Steroid hormones were analyzed in testes after extraction with diethyl ether. Decapsulated testes from neonatal and adult rats were placed in vials containing 100 and 500 µl water, extracted with 0.5 and 2.5 ml diethyl ether, respectively, and the procedure was repeated. Following evaporation, vials were added 100 and 500 µl diluent 1 (zero calibrator from PerkinElmer Life Sciences, Turku, Finland), respectively, and samples were vortexed and incubated for 10 min at 45 °C before analysis.

Ex vivo steroid production at GD 21 was determined as previously described (Vinggaard et al., 2005) except that a 5 h incubation was performed.

Gene expression levels determined by real-time RT-PCR

Tissues stored in RNAlater (paired testes, adrenals and ventral prostate) were homogenized, and total RNA was isolated using RNeasy-mini kit and RNase-Free DNase set from Qiagen (VWR International ApS, Albertslund, Denmark). Total RNA was eluted with 40 µl RNase-free water. RNA concentration and quality were determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA was synthesized from 1 µg total RNA using the Omniscript Reverse Transcription kit with T16 oligos and an 18S rRNA primer. Samples were incubated at 37 °C for 60 min followed by 60 °C for 10 min on PTC-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA, USA), and the final cDNA product was stored at –20 °C before quantification on the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Applera, Stockholm, Sweden) by standard TaqMan technology. Expression levels of the following genes were quantified in the testis and adrenals: scavenger receptor class B-1 (SRB1), steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage enzyme (P450scc) and 17α-hydroxylase/17,20-lyase (P450c17). The androgen receptor (AR) expression levels were quantified in the epididymides. In the prostates, expression levels were quantified for prostatic binding protein C3 (PBP C3), ornithine decarboxylase (ODC), testosterone-repressed prostate message 2 (TRPM-2), insulin-like growth factor I (IGF-I), complement component 3 (C3) and

androgen receptor (AR). For each sample, 2 µl cDNA (2.5 ng/µl) was amplified under universal thermal cycling parameters (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) in a total reaction volume of 20 µl. Three separate amplifications were performed for each gene, and, when intra-assay variation was above 10%, additional amplifications were performed. All genes were quantified from standard curves, and expression levels of each target gene were normalized to the expression level of the housekeeping gene 18S rRNA.

Expression of Desert hedgehog (Dhh), Patched 1 (Ptc-1), Insl-3 and SF-1 in the testis was measured separately by quantitative PCR using QuantiTect SYBR-Green PCR Kit (Qiagen) according to the manufacturer's instructions by using the DNA Engine Opticon system (MJ Research, Inc., Waltham, MA) with continuous fluorescence detection and normalized to the level of the housekeeping gene ribosomal protein S26 (S26).

Sequences for each primer set are listed in Table 1.

In vitro effects in the mouse Leydig tumor cell line mLTC-1

mLTC-1 cells (ATCC-CRL-2005) established from M548OP transplantable Leydig cell tumors carried in C57BL/6 mice were grown in 24-well culture plates (Costar, Corning, NY, USA) using the method previously described by Nikula et al. (1999). The cells were plated at a density of 1×10^5 cells/well, and prochloraz dissolved in DMSO was added to the cells in duplicates at 6.25, 12.5, 25, 50 and 100 µM. The incubation medium of control cells contained the same amount of DMSO (0.1%) as exposed cells. After 48 h of pre-incubation, the cells were washed with 2 ml PBS and stimulated with 0.135 IU/ml hCG (6500 IU/mg) kindly provided by The NIDDK's National Hormone and Pituitary Program and Dr. A.F. Parlow. After 3 h of incubation, the stimulation medium was removed and stored at -20°C until assayed for progesterone as described for the plasma samples. In parallel to the hCG-stimulated cells, other cells were added a resazurin solution to test for cytotoxicity as described (Laier et al., 2003). Medium from these wells (100 µl) was transferred to black microtiter plates (Costar, Corning, NY, USA) before fluorescence was measured.

In vitro effects in human adrenocortical carcinoma cell line H295R

H295R cells (ATCC, CRL-2128) established from the pluripotent human adrenocortical carcinoma cell line were grown in 24-well culture plates (Costar, Corning, NY, USA) containing 1 ml DMEM/F12 medium (GibcoBRL Life Technologies, Paisley, UK) supplemented with 2.0% Nuserum (BD Sciences Denmark), 1% ITS + premix (containing 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenium 1.25 mg/ml BSA and 5.35 µg/ml linoleic acid; BD Sciences, Denmark) and supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 250 ng/ml amphotericin B (Fungizone) at 37°C with a humidified atmosphere of 5% CO_2 /air. The cells were plated at a density of 2×10^5 cells/well and allowed to settle 1 day (approximately 24 h). Culture medium was removed, and new medium containing prochloraz dissolved in DMSO was added to the cells in triplicates at 0, 0.01, 0.03, 0.1, 0.3, 1 and 3 µM. The medium of control cells contained the same amount of DMSO (0.1%) as exposed cells. After 48 h of incubation, the medium was removed and stored at -20°C until assayed for progesterone and testosterone as described for the plasma samples. After exposure, the cells were incubated with a resazurin solution to test for cytotoxicity towards the H295R cells as described (Laier et al., 2003). Medium from these wells (200 µl) was transferred to black microtiter plates (Costar, Corning, NY, USA) before fluorescence was measured.

Statistical analyses

Statistical evaluation of pregnancy data and litter data. The litter was generally considered the statistical unit, and the alpha level was 0.05. The results were analyzed by analyses of variance (ANOVA) using the SYSTAT PC-version software package (Systat, 1990). In order to adjust for litter effects, litter was included in the analysis of variance as a nested factor.

Statistical evaluation of hormone data. A one-way ANOVA was employed for all groups and, if significant, followed by the post hoc test Dunnett's test. Significance was judged at $P < 0.05$.

Table 1
Sequences of each primer set used for quantitative real-time PCR

Gene	Primer set
18S	5'-FAM-ACC GGC GCA AGA CGA ACC AGA G-TAMRA-3'
rRNA	Forward, 5'-GCC GCT AGA GGT GAA ATT CTT G-3'
	Reverse, 5'-GAA AAC ATT CTT GGC AAA TGC TT-3'
SRB1	5'-FAM-AAA GCA TTT CTC CTG GCT GCG CAG-TAMRA-3'
	Forward, 5'-TCT GGT GCC CAT CAT TTA CCA-3'
	Reverse, 5'-AGC CCT TTT TAC TAC CAC TCC AAA-3'
StAR	5'-FAM-CTG ACT CCT CTA ACT CCT GTC TGC CTA CAT
	GGT-TAMRA-3'
	Forward, 5'-CCC TTG TTT GAA AAG GTC AAG TG-3'
	Reverse, 5'-TGA AAC GGG AAT GCT GTA GCT-3'
P450sc	5'-FAM-CCT TTA TGA AAT GGC ACA CAA CTT GAA GGT
	ACA-TAMRA-3'
	Forward, 5'-ACG ACC TCC ATG ACT CTG CAA T-3'
	Reverse, 5'-CTT CAG CCC GCA GCA TCT-3'
P450c17	5'-FAM-CGT CAA CCA TGG GAA TAT GTC CAC CAG A-
	TAMRA-3'
	Forward, 5'-GCC ACG GGC GAC AGA A-3'
	Reverse, 5'-CCA AGC CTT TGT TGG GAA AA-3'
AR	5'-FAM-TCG CGA TTC TGG TAT GCT GCT GC-3'
	Forward, 5'-GAC ACT TGA GAT CCC GTC CT-3'
	Reverse, 5'-GAG CGA GCG GAA AGT TGT AG-3'
PBP C3	5'-FAM-TCA TCT AGA ATA CTG CAG CCA GAA CCA CTG G-
	TAMRA-3'
	Forward, 5'-CCA TCC CCA TTT GCT GCT AT-3'
	Reverse, 5'-AGT CAC AGT TGA GTT AAT TGT ACC TCT AAT
	AAC-3'
ODC	5'-FAM-ACT CAC TGC TGT AAC ACA CAG CCT GTG CA-3'
	Forward, 5'-AAT GTG TGC AAG TAT CCC TTA CAG AA-3'
	Reverse, 5'-CAC AGC TTT GTA TCA TCC ACA TCT C-3'
TRPM-2	5'-FAM-AGT TTC TGA ACC AGA GCT CAC CCT TCT ACT
	TCT G-3'
	Forward, 5'-CTG GTT GGT CGC CAG CTA GA-3'
	Reverse, 5'-ATG CGG TCC CCG TTC AT-3'
IGF-1	5'-FAM-CAA CAC TCA TCC ACA ATG CCC GTC T-3'
	Forward, 5'-GAC CAA GGG GCT TTT ACT TC-3'
	Reverse, 5'-GCA GCG GAC ACA GTA CAT CT-3'
Compl C3	5'-FAM-CGT AGT CCA CTC CAG GCT CAC AAG-3'
	Forward, 5'-CAG CCT GAA TGA ACG ACT AGA CA-3'
	Reverse, 5'-AAA ATC ATC CGA CAG CTC TAT CG-3'
S26	Forward, 5'-AAGGAGAAACAACGGTCGTG-3'
	Reverse, 5'-GCAGGTCTGAATCGTGGTG-3'
Dhh	Forward, 5'-AAGCAACTTGTGCTCTGCT-3'
	Reverse, 5'-TCGTCCCAACCTTCAGTCAC-3'
Ptc-1	Forward, 5'-TACGTGGAGGTGTTTCATCA-3'
	Reverse, 5'-AACACCAAGGGCAAGAAATG-3'
SF-1	Forward, 5'-GTCTGTCTCAAGTTCCTCATCTC-3'
	Reverse, 5'-CTCGTTGCCCAATGCTTAT-3'
INSL-3	Forward, 5'-ACGCAGCCTGTGGACACC-3'
	Reverse, 5'-CAATCCGGGGTGTTCATT-3'

Statistical analysis of organ weights and morphological alterations. One to four males per litter at PND 16 were used in the analysis of terminal body weight and organ weights. Non-processed and ln-transformed data were examined for normal distribution and homogeneity of variance. In order to adjust for litter effects, litter was included in the analysis of variance as an independent, random and nested factor (proc mixed, SAS version 8, SAS Institute Inc, Cary, NC, USA). Organ weights were analyzed using body weight as a covariate. When an overall significant treatment effect was observed, two-tailed comparison was performed using least square means. In cases where normal distribution and homogeneity of variance was not obtained, data were additionally tested with the non-parametric Kruskal–Wallis test. The scoring of macroscopic lesions of external genitalia was analyzed using Fisher's Exact Test.

Results

Pregnancy and litter data

Prochloraz showed no effects on maternal weight during or after birth (Table 2). Maternal weight gain from GD 7 to GD 21 was significantly decreased at 150 mg/kg, but the maternal weight gain from GD 7 to PND 1 was not significantly affected by prochloraz. Litter sizes, birth weight of male and female offspring and sex ratios were unaffected. No effects were observed on pregnancy length, postnatal death or postimplantation–perinatal loss.

Anogenital distance and nipple retention

Prochloraz affected dose-dependently the anogenital distance in both male and female offspring (Table 2, Fig. 1A). At both 50 and 150 mg/kg, the male pups had significantly reduced anogenital distance compared to controls, whereas female pups had an increased anogenital distance compared to controls. Furthermore, both doses of prochloraz resulted in an increased number of nipples in male pups at PND 13 (Fig. 1B).

Hormone levels

Male fetuses from GD21 were sectioned, and levels of testicular testosterone and progesterone together with plasma testosterone, progesterone and luteinizing hormone (LH) were analyzed (Fig. 2). Testicular levels of testosterone were significantly decreased at both dose levels of prochloraz,

whereas levels of progesterone were significantly increased. Data on plasma pools from each exposure group showed a similar trend for both hormones. Testicular testosterone production *ex vivo* was examined at GD 21 as well, and, in agreement with the other data, a marked dose-dependent reduction was observed after both the low and the high dose prochloraz.

There was a tendency towards slightly increased LH plasma levels after prochloraz exposure, but, as the amounts of fetal blood were limited and only one pooled sample per group was available, it was not possible to test the hypothesis by doing statistical analysis. The abovementioned hormones were analyzed in serum and testes from male pups at PND 16 as well. A tendency towards reversed effects compared to the fetal effects was observed, i.e. increased levels of testicular and plasma testosterone and reduced levels of progesterone together with decreased LH plasma levels for the prochloraz-exposed pups (Table 3). The decrease in progesterone levels in males turned out to be statistically significant at 50 mg/kg.

Thyroid hormone levels were measured in blood plasma of PND 16 males and females, and a significantly reduced T4 level in prochloraz-exposed male pups (150 mg/kg) was observed (Table 3). However, T3 and TSH levels were not affected by prochloraz exposure for either sex.

Dysgenesis of external genitalia

Mild dysgenesis of the external genitalia was observed in males exposed to 50 mg/kg (mean score 1.25) and 150 mg/kg (mean score 1.28) prochloraz. In the controls, none of the males had scores ≥ 2 (mean score 1.0). In the rats exposed to 50 mg/kg prochloraz, 25% of the males had scores ≥ 2 , and 14% of the males had scores ≥ 2 in the group exposed to 150 mg/kg prochloraz. For both prochloraz groups, these effects were statistically significantly different from controls (Fisher's Exact Test).

Organ weight and histopathology

Body and organ weights of PND 16 male and female rat offspring are shown in Table 4. The number of pups (*n*) included for each group in the experiment varied as body weights and some organs were weighed for more than one pup per litter. A significantly diminished weight of both seminal vesicles and bulbourethral glands in male pups was observed at both doses. Female reproductive organs were unaffected. No dose-related histopathological effects were observed in any of the examined organs in males from GD 21 or PND 16.

Immunohistochemistry of fetal testes

Leydig cell cytoplasm was positively stained for P450c17, 17 β -HSD (type 10), StAR, P450scc and 3 β -HSD at GD 21. AR immunostaining was seen in the nuclei of Leydig cells and peritubular cells at GD 21. The intensity of P450c17 immunostaining was clearly increased in Leydig cells of prochloraz-exposed animals compared to controls (Fig. 3).

Table 2
Pregnancy and litter data

	Control	Prochloraz 50 mg/kg	Prochloraz 150 mg/kg
Number of litters	13	8	6
Maternal weight gain GD7 to GD21 (g)	93.2 \pm 9.4	95.4 \pm 12.9	77.8 \pm 12.6 ^a
Maternal weight after birth (g)	236.7 \pm 15.1	234.1 \pm 13.1	237.8 \pm 12.8
Maternal weight gain GD7 to after birth (g)	24.3 \pm 9.4	18.6 \pm 4.5	14.8 \pm 11.2
Pregnancy length (days)	22.5 \pm 0.5	22.9 \pm 0.6	22.8 \pm 0.8
Birth weight, (g) — males	6.3 \pm 0.4	6.4 \pm 0.4	6.5 \pm 0.5
Birth weight, (g) — females	6.0 \pm 0.3	6.1 \pm 0.2	6.2 \pm 0.5
Male pups (%)	46 \pm 14	41 \pm 9	44 \pm 13
Litter size at birth (live pups)	10.5 \pm 1.5	11.0 \pm 2.4	8.5 \pm 3.5
Postnatal death, mean	0.23 \pm 0.60	0.13 \pm 0.35	0.17 \pm 0.41
Postimplantation–perinatal loss (%) ^b	6.2 \pm 6.8	14.1 \pm 16.3	23.5 \pm 27.1
AGD in males, at birth (mm/cubic root of bw)	11.6 \pm 0.4	10.9 \pm 0.6 ^a	10.2 \pm 0.8 ^a
AGD in females, at birth (mm/cubic root of bw)	6.1 \pm 0.21	6.3 \pm 0.48 ^a	6.7 \pm 0.40 ^a
Body weight, PND 13 (g)—males	28.2 \pm 3.5	26.1 \pm 3.9	26.9 \pm 3.5
Body weight, PND 13 (g)—females	27.8 \pm 3.7	25.8 \pm 3.9	26.2 \pm 3.6

Data represent means \pm SD.

^a $P < 0.05$ compared to control group.

^b (no. of implantations – live pups at PND 6) / no. of implantations.

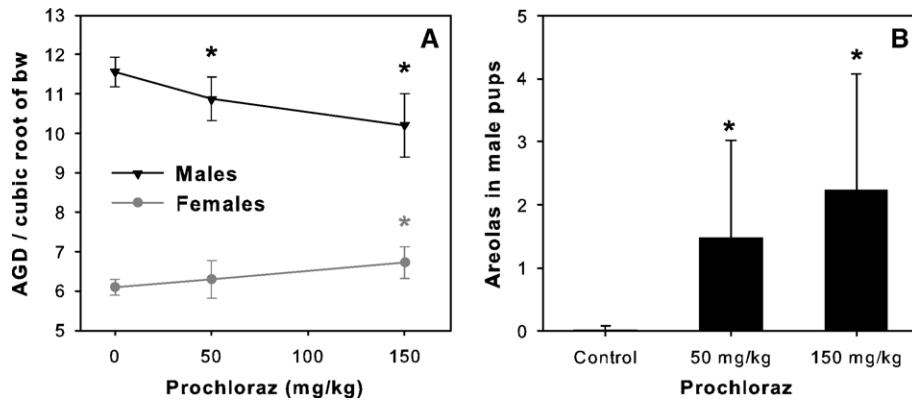


Fig. 1. (A) Effects on anogenital distance in male and female rat pups (PND 1) caused by perinatal exposure to 50 and 150 mg/kg prochloraz. AGD is shown as distance per cubic root of body weight. (B) Effects on nipple retention in prochloraz-exposed males (PND 13). The data represent the mean number \pm SD of areolas in male pups for $n = 13, 8$ and 6 for controls, 50 and 150 mg/kg, respectively. $*P < 0.05$.

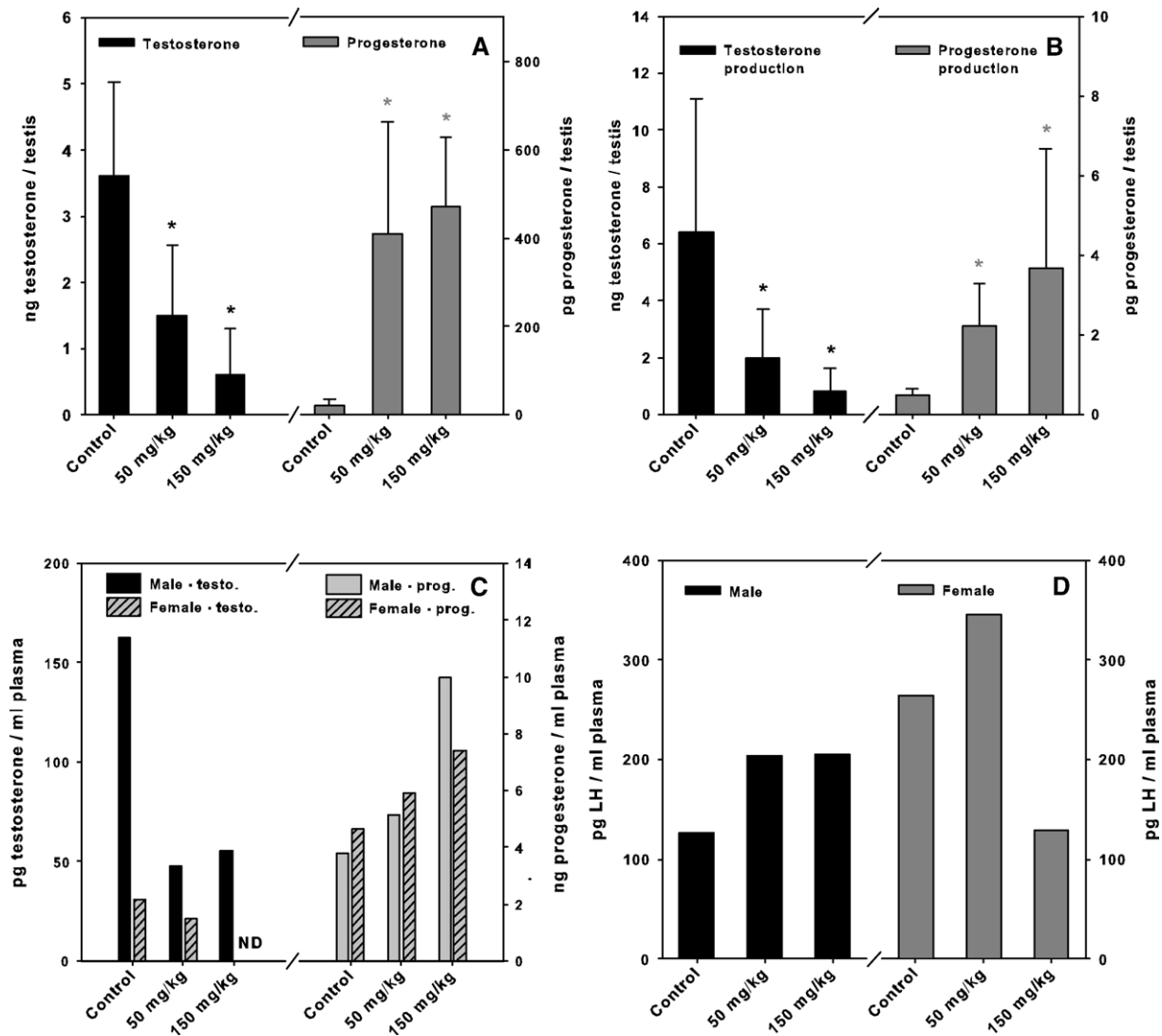


Fig. 2. Following in utero exposure to 50 and 150 mg/kg prochloraz, testicular levels of testosterone and progesterone (A) were measured in fetal rat testis at GD 21. Data are represented as litter mean \pm SD for $n = 6, 7$ and 5 for controls, 50 and 150 mg/kg, respectively. Testicular levels of testosterone and progesterone were measured in fetal rat testis after 5 h of incubation in cell culture medium and represented as litter mean \pm SD for $n = 6, 7$ and 5 for controls, 50 and 150 mg/kg, respectively (B). The data for plasma levels of testosterone and progesterone in male and female offspring (C) at GD21 represent the mean levels of total pooled samples (one pool for each group). Plasma levels of luteinizing hormone in male and female offspring at GD21 (D) are represented as the mean levels in total pooled samples (one pool for each group). $*P < 0.05$. ND: not determined.

Table 3
Hormones measured at PND 16 in male and female pups after perinatal prochloraz exposure

Testis		Control	50 mg/kg	150 mg/kg
Testosterone, ng/testis		0.30 ± 0.17	0.33 ± 0.28	0.54 ± 0.67
Progesterone, ng/testis		57.1 ± 23.2	47.7 ± 14.5	50.1 ± 41.0
Plasma	Sex	Control	50 mg/kg	150 mg/kg
Testosterone, pg/ml	M	67.7 ± 28.1	72.7 ± 74.0	123 ± 68.9
	F	56.3 ± 41.2	51.0 ± 26.9	32.3 ± 26.2
Progesterone, ng/ml	M	6.54 ± 1.85	3.77 ± 1.54 *	3.82 ± 1.51
	F	5.15 ± 1.84	3.98 ± 3.25	2.59 ± 1.24
Luteinizing hormone (LH), pg/ml	M	386 ± 382	225 ± 178	237 ± 207
	F	1605 ± 923	1257 ± 1413	2232 ± 889
Inhibin, nmol/l	M	0.88 ± 0.31	1.24 ± 0.47	1.03 ± 0.43
	F	0.78 ± 0.57	0.57 ± 0.41	0.45 ± 0.36
Triiodothyronine (T3), ng/ml	M	2.09 ± 0.13	2.13 ± 0.43	1.94 ± 0.11
	F	1.91 ± 0.23	1.91 ± 0.19	1.87 ± 0.20
Thyroxine (T4), ng/ml	M	127 ± 19.7	134 ± 27.8	97.3 ± 1.8*
	F	98.6 ± 12.7	91.4 ± 5.0	88.1 ± 11.1
Thyroid stimulating hormone (TSH), nmol/l	M	44.4 ± 20.9	47.8 ± 17.7	52.1 ± 25.7
	F	28.0 ± 23.0	21.4 ± 2.10	50.3 ± 13.0

Data represent means ± SD.

One-way ANOVA.

* Statistical significance compared to controls by Dunnett's t test ($P < 0.05$).

The intensity of immunostaining for 17 β -HSD (type 10) was reduced in prochloraz-exposed animals compared to controls (Fig. 4). No changes were observed in the intensity or localization of immunostaining for StAR, P450scc, 3 β HSD or AR.

Gene expression levels

Expression levels of genes involved in androgen biosynthesis and signaling and/or Leydig cell function were determined in paired testes and adrenals in male fetuses.

Prochloraz had no effect on the expression levels of the genes SRB1, StAR, P450scc and P450c17 in testis and AR in epididymides when compared to the housekeeping gene 18S at GD 21 or at PND 16 (Table 5). There was a clear tendency towards a dose-dependent up-regulation of P450c17 in fetal adrenals (GD 21) following exposure to 50 and 150 mg/kg prochloraz (Fig. 5). In PND 16, male pups expression of androgen-regulated genes was investigated in ventral prostates (Fig. 6). It was found that ODC, PBP C3 and IGF-1 mRNAs were significantly reduced in the prostates after exposure to 50 and 100 mg/kg prochloraz. In contrast, the expression levels of TRPM-2, Compl C3 and AR mRNAs were unaffected.

In vitro effects on steroidogenesis

In order to look for a suitable in vitro model that reliably detects compounds that affect steroidogenesis, a mouse Leydig cell line (mLTC-1) and a human adrenocortical carcinoma cell line (H295R) were investigated. In control and hCG-induced mLTC-1 cells, prochloraz caused a dose-dependent decrease of progesterone production (Fig. 7). Preliminary data indicate that this effect was seen irrespective of using cholera toxin, 8-bromo-cAMP or forskolin for stimulation of steroidogenesis (data not shown), indicating that the effect manifests itself at a level at or after protein kinase A activation. All stimulants caused elevated cAMP levels and consequently protein kinase A activation (data not shown).

H295R cells incubated with prochloraz showed a markedly dose-dependent reduction of testosterone production compared to control (Fig. 8). Testosterone levels were reduced by 50% with the lowest prochloraz concentration of 0.01 μ M, and the two highest concentrations (1 and 3 μ M) almost shut down the testosterone production. Progesterone levels were significantly increased after incubation with 0.1, 0.3, 1 and 3 μ M prochloraz.

Table 4
Body and organ weights for prochloraz-exposed male and female pups at PND 16

	n	Control	n	Prochloraz 50 mg/kg	n	Prochloraz 150 mg/kg
Males						
Body weights (g)	35	35.2 ± 4.3	25	32.2 ± 4.3	17	32.9 ± 4.9
Liver (g)	25	0.89 ± 0.16	15	0.85 ± 0.17	12	0.91 ± 0.19
Paired kidneys (g)	14	0.347 ± 0.053	8	0.321 ± 0.059	6	0.333 ± 0.067
Paired adrenals (mg)	25	10.5 ± 1.6	15	10.5 ± 1.5	12	10.5 ± 1.9
Right testis (mg)	35	66.0 ± 8.7	25	64.8 ± 8.7	17	61.9 ± 11
Left testis (mg)	35	66.4 ± 7.3	25	65.0 ± 8.2	17	62.6 ± 9.7
Paired epididymis (mg)	25	27.0 ± 5.3	14	25.1 ± 4.3	12	24.8 ± 4.5
Seminal vesicles (mg)	13	12.0 ± 3.1	8	8.78 ± 2.3 *	6	9.55 ± 1.5
Ventral prostate (mg)	25	16.5 ± 3.1	16	14.7 ± 3.5	12	13.0 ± 3.9
M. levator ani/bulbocavernosus (mg)	13	27.0 ± 6.3	8	22.2 ± 2.4	6	23.2 ± 1.9
Bulbourethral glands (mg)	13	2.16 ± 0.63	8	1.35 ± 0.47*	6	0.790 ± 0.59*
Females						
Body weights (g)	26	34.4 ± 4.3	9	31.5 ± 4.6	11	32.6 ± 4.6
Ovaries (mg)	26	6.09 ± 0.89	9	5.81 ± 1.4	11	6.33 ± 1.7
Uterus (mg)	26	24.9 ± 2.6	9	22.5 ± 2.9	11	23.8 ± 3.9

Data represent means ± SD.

One-way ANCOVA (body weight as the covariate).

* Statistical significance compared to controls ($P < 0.05$).

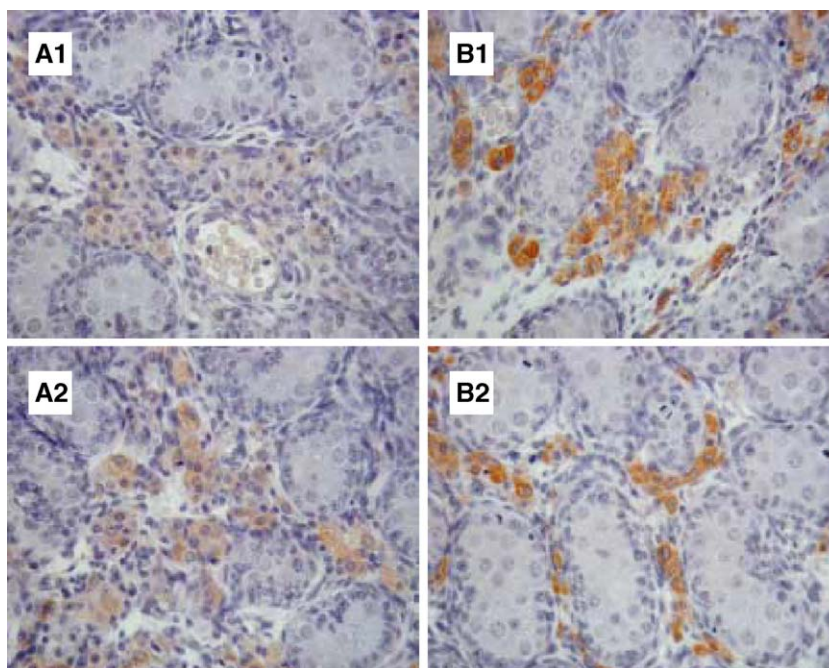


Fig. 3. (A1–2) Immunohistochemical staining of 17 α -hydroxylase/17,20-lyase in fetal control testis at GD 21. (B1–2) Immunohistochemical staining of 17 α -hydroxylase/17,20-lyase in fetal testis at GD 21 after exposure to 150 mg/kg prochloraz.

The formation of estradiol was significantly reduced at 0.1 μ M and concentrations above showing a dose-dependently correlation. Visual inspection and experiments to measure cytotoxicity by the resazurin method showed no cytotoxicity of prochloraz up to 3 μ M.

Discussion

In agreement with previous studies, prochloraz was found to feminize the male rat offspring by significantly reducing the anogenital distance after gestational exposure to 50 and 150 mg/kg. This feminization was further confirmed by the observation of nipple retention in the male pups at PND 13. These results are in agreement with recent results demonstrating a dose-dependent reduction of AGD in male offspring after gestational exposure to 125 and 250 mg/kg prochloraz (Noriega et al., 2005). However, this effect was found only for unadjusted AGDs, whereas significance was lost after adjustment for body

weight. In this study, the AGD was corrected by the cubic root of the body weight, and statistical significance was obtained at the low dose of 50 mg/kg. This dose may be close to NOEL as Vinggaard et al. (2005) did not observe any effects on AGD with 30 mg/kg prochloraz. The increased anogenital distance observed in female pups may indicate an androgenic potential of prochloraz in females. Initial in vitro studies of prochloraz did not indicate any androgen activity mediated via AR, but the disrupted fetal steroidogenesis causing high progesterone levels may be the causal factor of this virilization of the females (<http://progesterone.com/provera.html>).

Previously, perinatal exposure to 30 mg/kg prochloraz has been shown to significantly affect nipple retention (Vinggaard et al., 2005), and dose dependency has been demonstrated by Noriega et al. (2005), who found a LOEL at a dose of 125 mg/kg. As the exposure period in the latter study was GD 14–18 compared to our GD 7–PND 17, it suggests that the underlying causal events for the nipple retention observed at PND 13 has

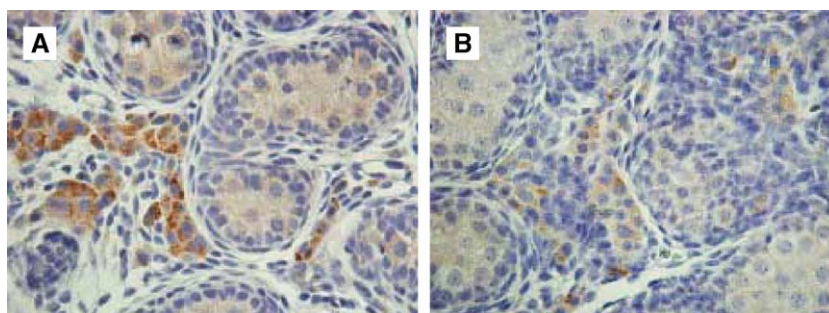


Fig. 4. (A) Immunohistochemical staining of 17 β -hydroxysteroid dehydrogenase (17 β -HSD) (type 10) in fetal control testis at GD 21. (B) Immunohistochemical staining of 17 β -hydroxysteroid dehydrogenase (17 β -HSD) (type 10) in fetal testis at GD 21 after exposure to 150 mg/kg prochloraz.

Table 5

Overview of effects of prochloraz on fetal steroidogenesis at gene and/or protein level

Proteins involved in Leydig cell function	Real-time RT-PCR (mRNA level)	Immunohistochemistry
SR-B1	↔	
StAR	↔	↔
CYP450 _{scc}	↔	↔
3 β -HSD		↔
17 α -hydroxylase/17, 20-lyase	↔	↑
17 β -HSD (type 10)		↓
Dessert hedgehog (Dhh)	↔	
Ptc-1 (Dhh receptor)	↔	
SF-1	↔	
INSL-3	↔	
AR	↔	↔

↔ unaffected, ↑ increased or ↓ decreased by perinatal prochloraz exposure.

taken place during gestation but that exposure during lactation may result in a lower LOEL of this parameter.

The decreased maternal weight gain during gestation caused by 150 mg/kg prochloraz suggests that prochloraz induced maternal toxicity or decreased weight of the uterine content, i.e. number and/or weight of fetuses or placentas or both. Neither the maternal weight gain from GD 7 to the first day after birth (PND 1) nor the number of offspring per litter was significantly affected by prochloraz, but both values seem lower at 150 mg/kg compared to control values. Therefore, the effect on maternal weight gain during gestation most probably reflects both slight maternal toxicity and slightly decreased litter size. But, overall, no pronounced maternal or fetal toxicity was evident at the selected doses.

The antiandrogenic potential of prochloraz has previously been demonstrated as it antagonized the androgen receptor in vitro (Andersen et al., 2002) and reduced weights of reproductive organs in the Hershberger assay (Vinggaard et al., 2002). These results classify prochloraz as an antiandrogen belonging to the same class of antiandrogens as vinclozolin, procymidone and *p,p'*-DDE, which primarily are considered to exert dihydrotestosterone (DHT)-dependent effects or affect DHT-dependent tissues (AGD, nipple retention, ventral prostate size, hypospadias) (Gray et al., 1999a). The decreased weight of the bulbourethral glands as well as the slightly feminized external genitals observed in male pups at PND 16 further supports that prochloraz affects DHT-dependent tissue. However, the significant effect on seminal vesicle demonstrates that prochloraz also affects reproductive tissue that is considered mainly testosterone-dependent, thus resembling the effects of the phthalates (Gray et al., 1999a).

In male fetuses, testosterone levels were significantly decreased in the testis, while progesterone levels were increased. Concomitantly, fetal plasma levels of testosterone and progesterone were reduced and increased, respectively. Similar results have been observed previously by Noriega et al. (2005) and Vinggaard et al. (2005) and advocate for inhibition of the androgen biosynthesis in the Leydig cells at steps subsequent to progesterone formation at the level of 17 α -

hydroxylase/17,20-lyase, 3 β -HSD and/or 17 β -HSD. This was supported by the markedly reduced ex vivo testosterone production in fetal testis at 150 mg/kg (10-fold). Previously, a dose of 30 mg/kg given in utero did not cause any significant effect on ex vivo testosterone formation (Vinggaard et al., 2005), but prenatal exposure to 250 mg/kg prochloraz given from GD 14–18 was shown to reduce fetal testosterone production at GD 18 (Wilson et al., 2004). Circulating levels of LH in fetal blood plasma at GD 21 tended to be increased in prochloraz-exposed male pups, indicating that the pituitary–Leydig cell axis was intact and effective, giving a negative feedback at this time point. The effects on the testicular and plasma hormone levels observed at GD 21 in the present study were reversible as these prochloraz-induced effects were absent at PND 16. In contrast a tendency towards a rebound effect on testosterone, progesterone and LH was seen.

Prochloraz has a well-documented effect on aromatase activity and the consequent metabolism of testosterone to estradiol (Andersen et al., 2002; Sanderson et al., 2002), but, since preliminary data showed no effect on testicular estradiol levels in prochloraz-exposed male fetuses, this mechanism of action probably plays no major role in the fetal testes.

We demonstrated for the first time that the levels of the thyroid hormone T4 in plasma at PND 16 were significantly decreased after exposure to prochloraz. A previous study showed that prochloraz reduced both T4 and TSH levels in castrated, testosterone-treated rats, indicating a direct effect at the CNS level (Vinggaard et al., 2002). In this study, TSH levels were not affected by prochloraz, but this discrepancy may reflect different responsiveness of castrated versus intact rats.

In general, no visible histopathological effects in the testes were found, but changes in the expression of two enzymes involved in synthesis or metabolism of steroids were detected by immunohistochemistry. In fetal testis, prochloraz induced an increased expression of the P450c17 enzyme, which is responsible for the hydroxylation of progesterone in the 17 α -position. This increased expression may reflect that the enzyme activity of P450c17 is inhibited which may further explain the accumulation of progesterone and marked reduction of

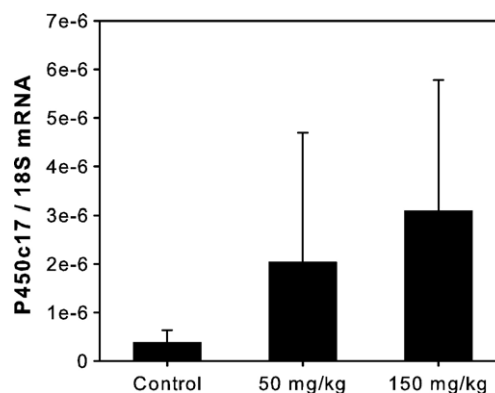


Fig. 5. Adrenal gene expression of cytochrome P450c17 determined by real-time RT-PCR in male fetal rats (GD 21) after exposure of 50 and 150 mg/kg prochloraz. The data represent the relative values of the mRNA levels of P450c17 and the housekeeping gene 18S.

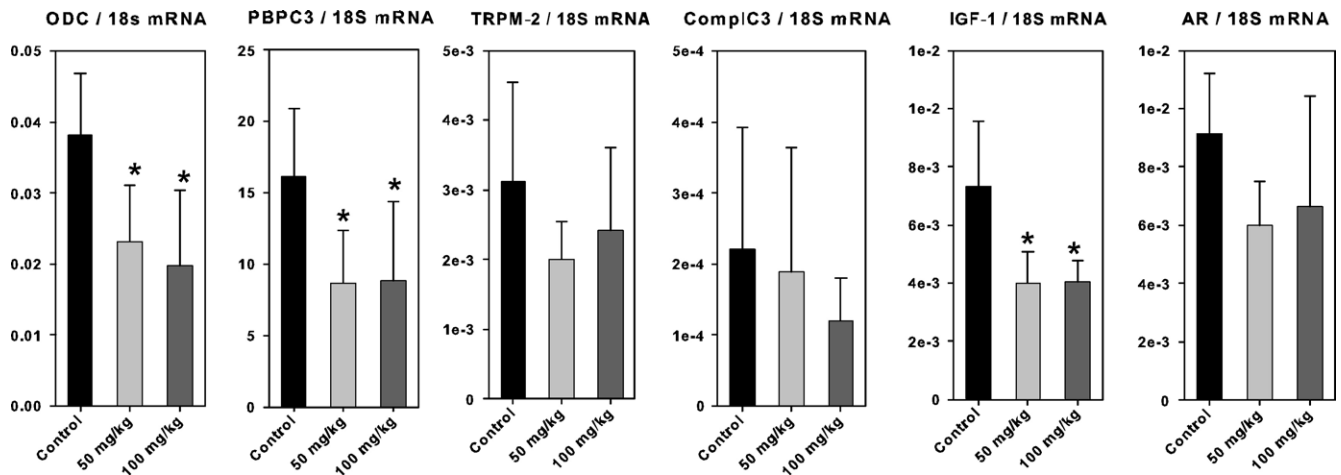


Fig. 6. Expression levels of androgen-regulated genes (ODC, PBP C3, TRPM-2, Compl C3, IGF-1 and AR) determined by real-time RT-PCR in ventral prostate from male rats (PND 16) after exposure to 50 and 100 mg/kg prochloraz. The data represent the values of the specific mRNA levels relative to the housekeeping gene 18S. The y axes represent arbitrary units, and therefore mRNA levels cannot be directly compared.

testosterone observed in male fetuses. Previous in vitro and in vivo studies of imidazole drugs such as ketoconazole suggest that the predominant effect on human adrenal and testicular steroidogenesis involves inhibition of P450c17 by direct interaction with the cytochrome P-450 component of the enzymes (Ayub and Levell, 1987, 1988). This is in agreement with the fact that we find an interaction at the protein level and not at mRNA level in the fetal testis. However, in the adrenals, an increased expression of P450c17 mRNA was seen, indicating that also steroidogenesis in adrenals was affected via a mechanism that differs from the one in the testes. Interestingly, current literature suggests that rat adrenals lack the expression of P450c17 (Pelletier et al., 2001). However, mRNA levels were clearly detectable and were significantly above the level of quantification for the analysis, although a general low expression was seen.

Concomitantly, immunohistochemical expression of 17 β -HSD (type 10) in fetal testis was reduced by prochloraz.

17 β -HSD (type 10) is a major product of both fetal and adult-type Leydig cells in rodents and seems preferentially to convert 3 α -androstenediol into dihydrotestosterone and estradiol into estrone. Thus, perinatal expression of this enzyme in fetal Leydig cells has been suggested to contribute to protection of these cells from estrogens and encourage androgen formation (Ivell et al., 2003; Mindnich et al., 2004). The reduced protein expression of 17 β -HSD (type 10) may indicate an increased activity of the enzyme that is induced as a secondary defense mechanism to protect the testis from the lowered androgen levels caused by prochloraz.

Although some in vivo effects of prochloraz on steroidogenesis such as the disturbance in testosterone levels and reduced weight of seminal vesicles may indicate a similar mechanism of action as the phthalates, no effects of prochloraz were observed on mRNA levels of SRB1, StAR or P450scc responsible for cholesterol trafficking and metabolism as reported for DBP (Barlow et al., 2003; Thompson et al., 2004). In male rat fetuses, the decreased testicular steroidogenesis caused by DBP has been demonstrated to precede the repressed transcription of StAR, Scarb1, P450scc and P450c17 and seems to be unique compared to the adrenals in which genes required for steroidogenesis were unaffected (Thompson et al., 2005).

The reduced expression levels of the androgen-regulated genes ODC, PBP C3 and IGF-1 observed in ventral prostate in PND 16 males after exposure to 50 and 100 mg/kg prochloraz further support the antiandrogenic effects of prochloraz and may be secondary to either AR antagonism or inhibition of testosterone formation. These findings are in agreement with previous results where expression of ODC and PBP C3 mRNA was found being reduced in ventral prostates of castrated testosterone-treated male rats treated with 50–150 mg/kg prochloraz (Vinggaard et al., 2002). Reduced expression of the IGF-1 gene has not previously been reported for prochloraz, but the fungicide fenarimol has been reported to reduce IGF-1 expression levels in a Hershberger assay (Vinggaard et al.,

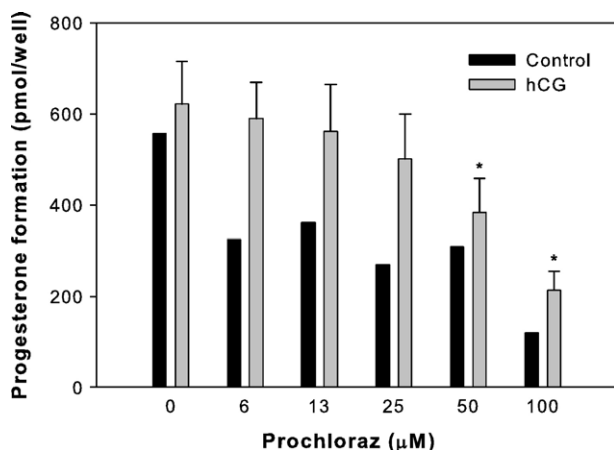


Fig. 7. In vitro effects of prochloraz on progesterone formation in mouse Leydig tumor cells (mLTC-1). Black columns represent unstimulated cells and the gray bars represent the hCG-stimulated cells. Data represent the mean \pm SD for a representative experiment in triplicate. The statistical analysis has been applied for the hCG-stimulated cells only. * $P < 0.05$.

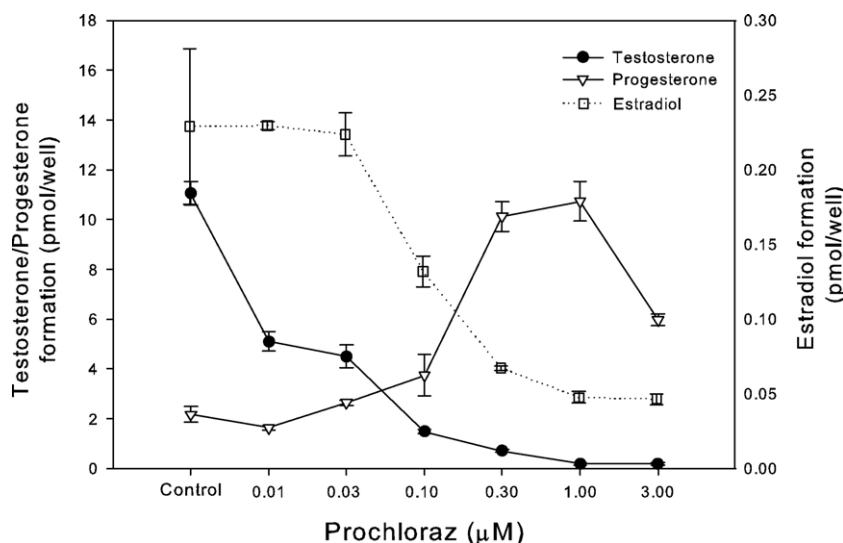


Fig. 8. In vitro effects of prochloraz on testosterone, progesterone and estradiol formation in human adrenocortical carcinoma cells (H295R). Data represent the mean \pm SD for a representative experiment in triplicate. * $P < 0.05$.

2005). In general, fenarimol and prochloraz have shown very similar profiles regarding their effects in the Hershberger assay (Vinggaard et al., 2005) indicating that prochloraz may also have the potential to affect IGF-1. Additionally, the absence of effect on TRPM-2 expression levels found in this study is in agreement with previous findings for prochloraz and fenarimol in the Hershberger assay (Vinggaard et al., 2005).

The effect of prochloraz on steroidogenesis was investigated in murine Leydig tumor cells (mLTC-1) and progesterone was measured as the endpoint as mLTC-1 cells synthesize testosterone in very limited amounts compared to androstenedione and progesterone. This is suggested to be due to the lack of isoform Type 3 of 17 β -hydroxysteroid dehydrogenase/17-ketoreductase, which is responsible for the reduction of androstenedione to testosterone (Panesar et al., 2003). Prochloraz was found to inhibit both basal and hCG-induced progesterone production in this cell line, the opposite effect on progesterone of what was seen in male fetuses in vivo. The reason for the reduced progesterone levels found in mLTC-1 cells is unknown, but obviously prochloraz affects steroidogenesis at a step before progesterone in these cells. It may be possible that prochloraz has more targets of action in the steroidogenic pathway affecting more than one step. Alternatively, the steroidogenic pathway in this cell line may deviate in more ways from physiological Leydig cells than we are aware of. Complementary investigation of steroidogenic effects of prochloraz was carried out with the human adrenocortical carcinoma cell line H295R that is an in vitro model capable of complete steroidogenesis. The results confirmed the testosterone reduction and progesterone increase observed in vivo and ex vivo with prochloraz and further supported that the disruptive effect of prochloraz on steroidogenesis is primarily mediated through a direct effect on the steroidogenic cells. Thus, this in vitro model may be a suitable candidate assay for future screening of compound-induced effects on steroidogenesis. Measuring additional metabolites in the androgen biosynthesis pathway

would be advantageous with this in vitro model for better understanding of the mechanisms of actions of prochloraz.

In conclusion, we have confirmed that prochloraz in addition to inhibiting AR also affects fetal steroidogenesis in male rats, suggesting that prochloraz's mechanisms of actions cannot be compared exclusively with classical antiandrogens such as AR antagonists or phthalates. Furthermore, an inhibitory effect on expression of androgen-regulated genes in ventral prostates and thyroid hormone levels was found in neonatal males. The result obtained in vivo using hormone, immunohistochemical and gene expression analyses together with the in vitro data indicates that the inhibitory effect on androgen biosynthesis of prochloraz is primarily due to inhibition of the 17 α -hydroxylase/17,20-lyase. In addition, an effect on androgen metabolism may play a role as the protein expression of 17 β -HSD (type 10) was found being up-regulated. These results are important for future assessment of pesticides and other environmental contaminants with anti-androgen potential.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.taap.2005.10.013.

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Appendix Y – Additional contributions

Posters

Hormonal and testicular effects of DINP and DEHP in fetal male rats.

Miniposter at 13th European Testis Workshop, April 24-28, 2004, Dunblane, Scotland, UK.

Diisobutyl phthalate has comparable antiandrogenic effects to Di-n-butyl phthalate in fetal rat testis.

Poster at 45th Annual meeting of the Society of Toxicology, San Diego, March 5-9, 2006.

Additional results

In vitro study on PPAR α and PPAR γ transactivation by environmental contaminants

In vivo study on butylparaben, PFOA and rosiglitazone

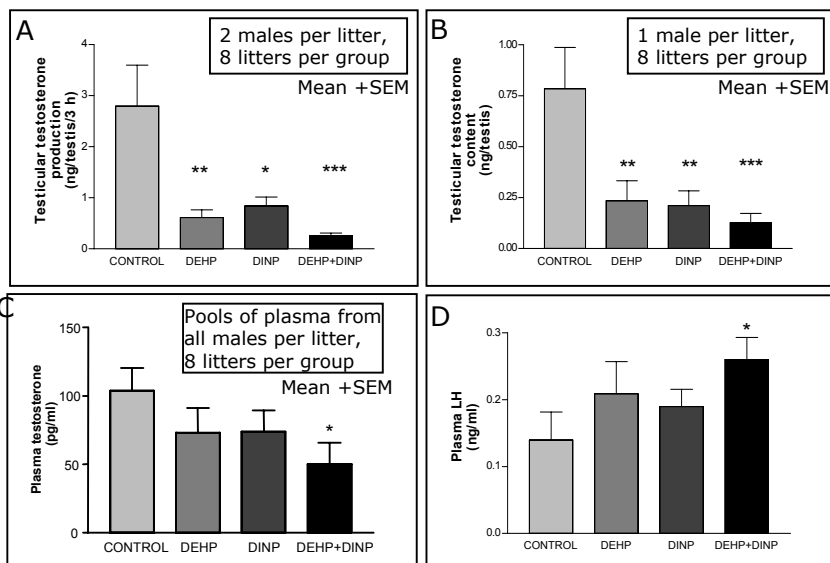
Hormonal and testicular effects of DINP and DEHP in fetal male rats

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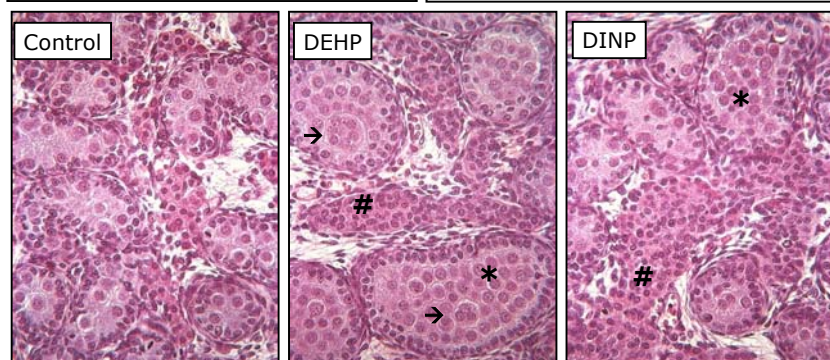
Danish Institute for Food and Veterinary Research, Mørkhøj Bygade 19, 2860 Søborg, Denmark.

INTRODUCTION: As the plasticizer DEHP (diethylhexyl phthalate) is known to be testis toxic, DINP (diisononyl phthalate) has been suggested as a substitution. **MATERIALS & METHODS:** In this study, 4 groups of 8 pregnant Wistar rats were dosed by gavage from gestation day (GD) 7 to 21 with either vehicle, 300 mg DEHP/kg bw/day, 750 mg DINP/kg bw/day or with a combination of 300 mg DEHP/kg bw/day + 750 mg DINP/kg bw/day. Dams were euthanized at GD 21 and testes were removed from male fetuses. Testes were either used for analysis of testosterone production, testicular testosterone content or for histopathology. See detailed method description in Borch *et al.*, 2003.



RESULTS & DISCUSSION:

Testicular testosterone production (A), testicular testosterone content (B) and plasma testosterone content (C) was reduced by DEHP and DINP, and there was a tendency to further reductions in the combination group. Plasma LH (D) was upregulated by DEHP +DINP. DEHP is more potent than DINP, as the effects of DEHP was similar to or stronger than the effects of DINP, even though the DEHP dose was lower than the DINP dose. The stronger effect of the combination than each compound alone points to an additive effect.



Histopathological effects of DEHP appear to be more abundant than effects of DINP, as listed in the table below. Multinucleated germ cells were seen in most testes from DINP and DEHP groups. Leydig cell effects and chords with enlarged diameter and many germ cells were more abundant with DEHP than DINP exposure. * Chords with increased diameter and many germ cells
→ Multinuclear germ cells
Leydig cell clusters

	Control	DEHP 300	DINP 750	DEHP+DINP
Multinuclear germ cells	0%	100%	83%	95%
Enlarged seminiferous chords containing many germ cells	0%	68%	22%	76%
Hyperplasia of Leydig cells with reduced cytoplasm	18%	72%	52%	70%

CONCLUSIONS & PERSPECTIVES:

- DINP is less potent in inducing hormonal and testicular effects than DEHP
- Germ cell effects appear to be highly abundant. In the DINP group multinucleated germ cells appear to be an "early" effect present in testes without enlarged/ germ cell rich seminiferous chords or apparent Leydig cell effects
- Stronger effects are seen with combined exposure to these two similarly acting compounds. Will combination of very low doses of many phthalates and other compounds with similar effects have effects relevant for humans?

Diisobutyl phthalate has comparable anti-androgenic effects to Di-*n*-butyl phthalate in rat fetal testis

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Introduction

PHthalates are widely used as plasticizers in various consumer products and building materials. Some of the phthalates are known to interfere with male reproductive development in rats, and di-*n*-butyl phthalate (DBP), diethylhexyl phthalate (DEHP) and butyl benzyl phthalate (BBP) were recently banned for use in toys in the EU due to their reproductive toxicity. Diisobutyl phthalate (DiBP) has similar structural and application properties as DBP, and is being used as a substitute for DBP. However, DiBP has not been as widely investigated in experimental animals as DBP.

Diisobutyl phthalate (DiBP) (see fig 1.) has not previously been examined in fetal rat testis, though it is widely used and suspected of having similar reproductive effects as the well-studied DBP. Due to its similarities to DBP, DiBP can be used as a substitute for DBP, and is presently used in PVC, inks, paints, adhesives and cosmetics. Around 100 tons of DiBP is used in DK per year mainly as softeners in plastic, rubber, paints and glue, which account for 95% of the use. In the US, the monoester metabolite of DiBP, mono-isobutyl phthalate (MiBP), was found in women's urine in levels ranging from 0.7 ng/mL (25th percentile) to 5.1 ng/mL (75th percentile) (Swan *et al.*, 2005). The exposure level of children to DiBP is unknown, but an estimate of the total exposure to DBP in Danish children aged 1-6 years is 400 µg/kg bw/day. This age group is estimated to be the group with the highest exposure level of DBP (Müller *et al.*, 2003).

Since 1999 the six phthalates DEHP, DBP, BBP, DiNP, diisododecyl phthalate (DiDP) and di-*n*-octyl phthalate (DnOP) have been temporarily banned in the EU in the manufacture of toys and childcare articles for children under the age of three because of their carcinogenic, mutagenic and reprotoxic effects. Recently, DBP has along with DEHP and BBP been banned for use in toys irrespective of age categories due to their reproductive toxicity. DiNP, DiDP, and DnOP were concurrently banned in toys that can be put in the mouth by children.

Objectives

- To investigate if DiBP has comparable antiandrogenic effects to DBP after prenatal exposure.
- To determine the optimal time for detection of effects on fetal testosterone production.

Materials and Methods

- Pregnant Wistar rats were gavaged from GD 7 to GD 19 or GD 20/21
- Doses: Vehicle (corn oil) or 600 mg DiBP/kg bw/day
- Male fetuses were examined at GD 19 or GD 20/21
- AGD in males and females was measured
- Testicular testosterone level and production were investigated
- Quantitative RT-PCR: investigation of mRNA expression of the steroidogenic factors SR-B1, PBR, StAR, P450scc, and P450c17 (see fig 2.).
- Immunohistochemical studies of StAR, and P450scc protein levels in fetal testes.

Fig 1. Structure of diisobutyl phthalate

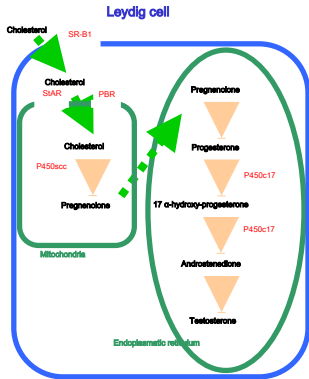
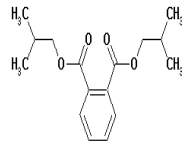


Fig 2. The steroidogenic pathway

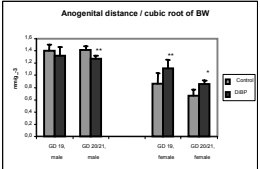


Fig 3. Anogenital distance (cubic root of BW) at GD 19 and GD 20/21 in male and female rat. Figure show mean ± SEM. Asterisks indicate a statistically significant difference compared to controls ***p<0.05, **p<0.01.

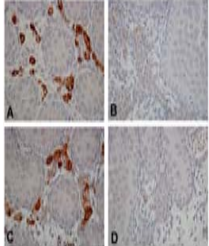
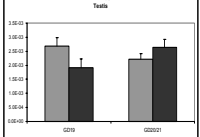
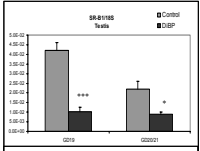


Fig 6. Immunohistochemical staining of testes from GD 19 (A+B) and GD 20/21 (C+D) in male rat fetuses exposed to vehicle (A+C) or 600 mg DiBP/kg bw/day (B+D) from GD 7 to GD 19 or GD 20/21. DiBP-exposed animals had a larger number of gonocytes, which were located centrally in the seminiferous tubules (*) due to vacuolization of Sertoli cell cytoplasm (B). Leydig cells (*) of DiBP-exposed animals were found in clusters and had small cytoplasm and small irregular nuclei. > Multinucleated gonocytes. Original magnification 40x.

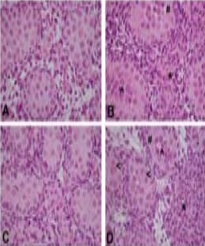


Fig 7. Testicular histopathology at GD 19 (A+B) and GD 20/21 (C+D) in male rat fetuses exposed to vehicle (A+C) or 600 mg DiBP/kg bw/day (B+D) from GD 7 to GD 19 or GD 20/21. DiBP-exposed animals had a larger number of gonocytes, which were located centrally in the seminiferous tubules (*) due to vacuolization of Sertoli cell cytoplasm (B). Leydig cells (*) of DiBP-exposed animals were found in clusters and had small cytoplasm and small irregular nuclei. > Multinucleated gonocytes. Original magnification 40x.

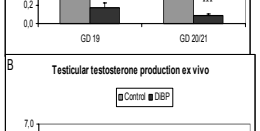
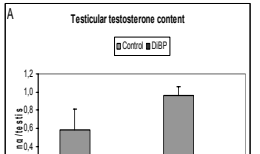


Fig 4. Testosterone levels at GD 19 and GD 20/21 in male rat fetuses. (A) Testicular testosterone content (B) Testicular testosterone production ex vivo (mS-6). Figures show mean ± SEM. Asterisks indicate a statistically significant difference compared to controls ***p<0.001.

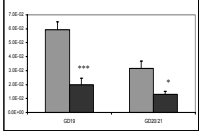
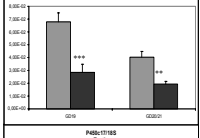
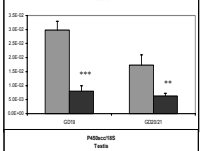


Fig 5. Expression of SR-B1, PBR, StAR, P450scc and P450c17 at GD 19 and GD 20/21 in testis relative to 18S rRNA. Data for mRNA levels are arbitrary and represent means ± SEM (mS-6). *p < 0.05; **p < 0.01; ***p < 0.001.

Results

- AGD in males was significantly reduced at GD 20/21 and AGD in females was significantly increased at GD 19 and GD 20/21 after DiBP exposure (Fig 3)
- At GD 19 and GD 20/21, testicular testosterone content and testicular testosterone production *ex vivo* were reduced in the DiBP group compared to controls (Fig 4)
- Gene expression levels of SR-B1, StAR, P450scc and P50c17 were decreased at GD 19 and GD 20/21. No effect was observed on PBR mRNA (Fig 5)
- Reduced immunohistochemical staining intensity of the two proteins StAR and P450scc was seen with DiBP (Fig 6)
- DiBP exposed animals had the histopathological characteristics also seen with other phthalates at GD 20/21. The number of gonocytes appeared to be increased, and they were located centrally in the seminiferous tubules due to vacuolization of Sertoli cell cytoplasm. Leydig cells were found in clusters and had small cytoplasm and small irregular nuclei (Fig 7)

Conclusions

- Based on the current results, it appears likely that DiBP has similar reproductive effects to DBP, such as testicular and epididymal atrophy or agenesis, and reduced semen quality.
- DiBP has similar testicular and developmental effects as DBP and DEHP, indicating a reason for concern about the use of DiBP as a substitute for DBP.
- In this study, GD 20/21 appears to be the optimal time for investigating changes in anogenital distance, testosterone levels, and testicular histopathology. At the m-RNA level, effects on factors involved in steroidogenesis seems more detectable at GD 19.

In vitro study on PPAR α and PPAR γ transactivation by environmental contaminants

Peroxisome proliferators activated receptors (PPARs) are a family of nuclear receptors (α , γ 1, γ 2, and δ , previously called β), which are present in many tissues. PPARs have mainly been examined for their involvement in fatty acid metabolism in liver, where PPAR α is involved in the catabolism of fatty acids, and PPAR γ promotes storage of fatty acids ¹. PPARs promote the uptake of cholesterol in hepatic cells, and may thereby reduce the cholesterol content of extrahepatic tissues. PPAR γ ligands are used as hypolipidemic and anti-diabetic drugs. PPARs also play a role in the immune system, and PPAR γ ligands have also been found to have an anti-tumor effect (breast, prostate), and have been tested in clinical trials for prostate cancer treatment ².

The natural ligands for PPARs have not been identified, although many endogenous compounds have been found to bind the receptors with varying affinity. Whether any of the various potential ligands may be a true natural ligand depends on the affinity of the ligand as well as the physiological concentration of the compound in various cell types. 15-deoxy-delta12,14-prostaglandin J2 has been suggested as an endogenous ligand for PPAR γ , while mono- and polyunsaturated fatty acids, long-chain fatty acyl-CoAs as well as eicosanoids have been suggested as natural PPAR α ligands ³. The anti-diabetic drugs thiazolidinediones are high-affinity exogenous PPAR γ ligands and the hypolipidemic fibrates are specific PPAR α ligands ³.

Additionally, several environmental contaminants have been found to be PPAR ligands in vitro. Peroxisome proliferation is a common toxicological response, which is related to chemically induced alteration of hepatic function. Several environmental contaminants have been described as peroxisome proliferators based on their hepatic effects, but have not been tested for PPAR interaction in vitro. Certain perfluorinated compounds, e.g. perfluorooctanoate (PFOA) and perfluorooctanesulfonate (PFOS), which are used for surface treatment and emulsification, are known to be peroxisome proliferators. PFOA has been found to activate PPAR α in vitro, whereas the interaction of PFOS with PPARs has not been examined ^{4,5}. Additionally, metabolites of the abundantly used phthalates, which are present in PVC and other consumer products, are known to activate all types of PPARs ⁶. Certain pesticides have also been reported to activate PPARs ⁷.

In addition to the hepatotoxic effect, some exogenous PPAR agonists including PFOA and phthalates are found to be testicular toxicants and/or suppress steroid synthesis in testes and ovaries of experimental animals ⁸⁻¹⁰. Certain phthalates reduce testosterone production in rats, and it has been suggested that phthalate exposure is related to reproductive disorders in humans ¹¹. As various

thiazolidinedione drugs also affect steroid synthesis in vitro and in vivo in animals and humans, it may be hypothesized that PPARs are somehow involved in chemically-induced inhibition of steroid synthesis, as reviewed by Corton & Lapinskas¹⁰.

Human blood levels of PFOA, PFOS and similar compounds have increased since the 1970s, but due to their accumulation in the environment, these compounds have recently been phased out by the manufacturer and blood levels are now levelling out¹². Humans are exposed to high levels of phthalates mainly via food, as phthalates present in the environment end up in e.g. water, fish, and plants, but to some extent children are also exposed via mouthing of toys or via inhalation of dust containing phthalates¹³.

In addition to phthalates and perfluorinated compounds, studies have shown testosterone-reducing and reproductive toxicity of parabens, which are widely used in moisturizers and cosmetics. Rat studies showed reduced testosterone levels and sperm production when orally exposed to butylparaben or propylparaben, while methylparaben and ethylparaben did not affect hormone levels or sperm production¹⁴⁻¹⁶. As humans may be exposed to high levels of these chemicals, it is essential that their toxicological profiles are examined thoroughly. Parabens are absorbed from skin and the gastrointestinal tract, hydrolysed, conjugated and excreted via urine and can be detected in urine of humans from the general population¹⁷. Parabens were also found in human breast tumors, and as parabens act as weak estrogens, this finding has led to concern for their involvement in breast cancer development^{18,19}.

Prochloraz is a fungicide with antiandrogenic effects in rats and multiple mechanisms of action including antagonism of the androgen receptor and inhibition of fetal testosterone synthesis²⁰.

In the current study, propylparaben, propylparaben, PFOA, monoethylhexyl phthalate (MEHP), and prochloraz were evaluated for their ability to interfere with PPAR α and PPAR γ activation or antagonism in a reporter gene assay along with the PPAR α ligand WY14,643 and the PPAR γ ligand rosiglitazone.

MATERIALS AND METHODS:

Test compounds: Rosiglitazone (BRL 49653), cas no. 122320-73-4, was from Cayman Chemicals (cat no 71740). WY 14,643, cas no. 50892-23-4, was from Calbiochem (cat no 681725). Propylparaben, cas no. 94-26-8, purity 99+%, was from Acros, Belgium. Propylparaben, cas no. 94-13-3, purity 99+%, was from Acros, Belgium. Perfluorooctanoate (PFOA), cas no. 335-67-1, purity 96%, was from Acros, Belgium. Monoethylhexyl phthalate (MEHP), cas no. 4376-20-9 was from

Accustandard, New Haven, CT. Prochloraz, ca no. 67747-09-5, purity 99.3%, was from Institute of Organic Chemistry, Warsaw, Poland. Test compounds were dissolved in DMSO (Sigma) to concentrations of 1 mM (Rosiglitazone) or 30 mM (other compounds). NIH-3T3 cells and plasmids containing the PPAR respondent UAS-TK-luc reporter as well as gal4-DBD_mPPAR α LBD and gal4-DBD_mPPAR γ LBD expression vectors were kindly provided by S. Mandrup, University of Southern Denmark.

NIH-3T3 cells were maintained in phenol-free DMEM/F12 (Gibco, Paisley, UK) with 10% charcoal-filtered calf serum (Biological Industries Ltd, Israel) and 1% PSF at 37°C in 5% CO₂/air. To examine the effect of different compounds on ligand-induced PPAR transactivation, cells were transiently transfected with the reporter construct 4xUAS-TK-luc and vectors expressing either PPAR α or PPAR γ . As positive controls WY14,643 was selected as a potent ligand for PPAR α and rosiglitazone as a potent ligand for PPAR γ .

On day 1 of the experiment, cells were counted and transferred to 96-well cell-culture plates (PerkinElmer, Massachusetts, USA) at a density of 7000 cells per well. After 20 hours, cells were transfected with UAS-TK-luc reporter plasmid and either gal4-DBD_mPPAR α LBD or gal4-DBD_mPPAR γ LBD expression vectors using FuGene ® (Roche, Mannheim, Germany) as a transfection reagent. One hour before transfection, medium was changed; FuGene and serum-free medium was mixed using 4.5 μ l FuGene in 10 μ l medium per well and added to a mixture of the plasmids with a total cDNA content of 75 ng per well. Plasmids were mixed in a 1:2 ratio for gal4-DBD_mPPAR α LBD and 4xUAS-TK-luc and a ratio of 1:1 for gal4-DBD_mPPAR γ LBD and 4xUAS-TK-luc. These ratios and cDNA concentrations had been selected as the optimal for each receptor giving the highest activation level compared to other plasmid ratios and DNA contents. The FuGene-cDNA mixture was incubated for 30 minutes before being added to the cells.

5 h after transfection, media was removed and new media containing test compounds was added and left for incubation over night (22 h). Concentrations of test compounds ranged from 0.3 to 100 μ M and the maximal concentration of the solvents DMSO or ethanol were below 0.3%, which had previously been found not to be cytotoxic. Cells were lysed in a lysis buffer containing 25 mM trisphosphate, pH 7.8, 15% glycerol, 1% Triton X-100, 1mM DTT, and 8 mM MgCl₂, followed by shaking for 15 min. Luciferase activity was measured directly using a BioOrbit Galaxy luminometer by automatic injection of 40 μ l substrate containing 1 mM luciferin and 1 mM ATP in lysis buffer and the chemiluminiscence generated from each well was measured over a 1 s interval after an incubation time of 2 s.

In the agonist assay, the luciferase activity levels induced by test compounds were compared with the luciferase activity level in wells containing media only. Results are shown as fold induction compared to the activity levels obtained with the specific ligands. In the antagonist assay, the test compounds were added to wells together with either 30 μ M WY14,643 (PPAR α assay) or 1 μ M rosiglitazone (PPAR γ assay). Results are shown as percentage activation compared to the activity level obtained with the same concentrations of WY14,643 or rosiglitazone alone.

Each plate included three wells for each concentration of test compounds, 12 wells of the negative control (media only) and 12 wells per plate of positive controls (WY14,643 or rosiglitazone). All assays were performed in triplicates and if variation between the runs was too large, a fourth or fifth run was performed. Results are shown as mean \pm SD of the three independent experiments.

Cytotoxicity was measured after incubation with test compounds using Alamar Blue $\text{\textcircled{R}}$ (BioSource, Camarillo, CA) to assess the proliferation of cells. Cells were grown under similar conditions as for the reporter gene assay, except that black 96-well microtiterplates (Costar, Corning, NY) were used. Alamar Blue was then added to media in a final concentration of 10%. Fluorescence levels increase as Alamar Blue is reduced by active cells, and the measured fluorescence thus reflects the number of living cells. After the addition of Alamar Blue, fluorescence levels were monitored for 4 h using a Victor2, 1420 Multilabel counter (PerkinElmer, Massachusetts, USA). Fluorescence levels were increasing approximately linearly from 2 to 4 h incubation time, and the cytotoxicity at different concentrations of test compounds were compared after 3 h of incubation. Results are shown as mean \pm SD of the three independent measurements.

Statistical analysis was performed using SAS Enterprise guide 3.0 (SAS Institute Inc). Non-processed and ln-transformed data were tested for homogeneity of variance and normal distribution. Using the appropriate transformation, data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test.

RESULTS:

Transactivation of PPAR α and PPAR γ was examined in reporter gene assays. For the determination of agonism, transactivation of PPAR α and PPAR γ was assessed by adding different concentrations of test compounds to cells transiently transfected with expression plasmids for either mouse PPAR α or mouse PPAR γ and a reporter containing the PPAR response element coupled to the luciferase gene (UAS-TK-luc).

Concentration-response curves for the control ligands WY14,643 and rosiglitazone were determined in order to select concentrations relevant for the determination of antagonism. Sub-maximal activation (60 to 75 % of the maximal observed activity) on the steep linear part of the curve was obtained with 30 μ M WY14,643 and 1 μ M rosiglitazone (Fig. 1). These concentrations were applied for antagonist assays, in which the positive control ligands were added together with the test compounds. The concentration-response curve for rosiglitazone only included concentrations up to 10 μ M as higher concentrations resulted in high concentrations of DMSO (above 0.3%), which were cytotoxic to the cells.

Butylparaben and propylparaben were weak activators of PPAR α with lowest observed effect concentrations (LOEC) of 30 μ M and 100 μ M, respectively, and transactivated PPAR γ from 10 and 100 μ M, respectively (Fig. 2). Both parabens activated PPAR α approximately 2-fold at 100 μ M. Maximal induction of PPAR γ by 100 μ M of butylparaben was approximately 4-fold, while 100 μ M propylparaben activated PPAR γ 2-fold. PFOA was a strong activator of PPAR α from 30 μ M with a maximum of 15-fold activation at 100 μ M (Fig. 2A). PFOA was a weaker activator of PPAR γ from 30 μ M with a maximum of 4-fold activation at 100 μ M (Fig. 2B). MEHP activated PPAR α from 3 μ M and PPAR γ from 10 μ M (Fig. 2). MEHP was a much stronger activator than the other compounds with a maximal activation at 100 μ M of 28-fold for PPAR α and 49-fold for PPAR γ . Prochloraz reduced the luminescence level compared to controls in both the PPAR α - and the PPAR γ -assay (Fig.2). This may either indicate cytotoxicity or antagonism of a “background” activation of PPARs by factors present in the media.

In the antagonist assays, test compounds were added concomitantly with the relevant control ligand. Propylparaben, propylparaben, and prochloraz reduced the luciferase activity measured in the PPAR α as well as the PPAR γ assay (Fig. 3). However, the Alamar Blue assay revealed cytotoxicity at high concentrations of these compounds (Fig. 4). As the concentration-response curves for the PPAR α and PPAR γ antagonist assays were very similar to the curves in the Alamar Blue assay, this indicates that the alterations in the antagonist assays were in part due to cytotoxicity. On the other hand, some degree of antagonism probably occurs, since low luminescence levels were seen at low (non-cytotoxic) concentrations of propylparaben in the PPAR γ antagonist assay and at relatively low concentrations of prochloraz in the PPAR α as well as the PPAR γ antagonist assays (Fig. 3).

The highest concentrations of MEHP and PFOA increased the measured luciferase activity in the PPAR α antagonist assay, but also in the Alamar Blue assay, indicating that the compounds stimulated the proliferation of cells (Fig. 3A and 4). In the PPAR γ antagonist assay, PFOA did not

alter luciferase activity, whereas MEHP reduced luminescence at 30 μ M. This likely reflected the cytotoxicity of MEHP, as the Alamar Blue assay showed that MEHP was cytotoxic at 30 μ M, but stimulated cell proliferation at 100 μ M.

Also 30 μ M WY14,643 increased measured fluorescence in the Alamar Blue assay to approximately 120% of the level observed in wells containing media only (data not shown). This indicates stimulation of cell proliferation. When 30 μ M WY14,643 or 1 μ M rosiglitazone were added to wells together with test compounds in the Alamar Blue assay, the results were similar to the results without these control ligands (data not shown). As the activation by PFOA, MEHP, WY14,643 and rosiglitazone observed in the PPAR agonist assays was several-fold, it is clear that this was not solely due to the stimulation of cell proliferation.

Results are collected in Table 1 and compared to findings described in the literature.

DISCUSSION:

This is the first study to describe parabens as being PPAR ligands. The transactivation of PPAR α and PPAR γ by butylparaben and propylparaben is weaker compared to the activation seen with compounds such as MEHP and PFOA. Both compounds appeared to be slightly stronger activators of PPAR γ than PPAR α as judged by their lowest observed effect concentrations and their maximal fold-induction, but this may also reflect a higher sensitivity of the PPAR γ assay in general. The cytotoxicity observed with high concentrations of butylparaben and propylparaben may in fact lead to an underestimation of the potency of these compounds. Analysis of paraben interaction with PPARs using other (not cell-based) methods may therefore reveal higher potency than observed with these cells.

The concentration-response curves show that butylparaben and prochloraz antagonize the activation of PPAR γ obtained with rosiglitazone, and prochloraz also antagonizes the activation of PPAR α by WY14,643. The impact of this antagonism on the physiological function of endogenous PPAR ligands is unknown. PPARs influence various physiological pathways including reproductive hormone synthesis, fatty acid metabolism, insulin function, immunology, cancer development, the central nervous system and the cardiovascular system². Therefore, the observation that parabens are PPAR ligands indicates that these compounds may have multifaceted effects – beneficial or adverse. Other parabens than those investigated in the current study are abundantly used as well and further studies may reveal whether they also activate PPARs.

Several studies have described the activation of PPAR α and PPAR γ by MEHP and PFOA. The activation of PPARs by PFOA and MEHP in the current study is comparable to results described by

other authors. As listed in Table 1, PPAR α activation is obtained at a higher concentration of PFOA in the current study than in the study by Maloney & Waxman (1999) indicating a higher sensitivity in their assay compared to ours. On the other hand, PPAR γ activation by PFOA could not be detected in that study, but was seen in our assay. Furthermore, our assay detects PPAR α and PPAR γ activation by MEHP at low concentrations compared to the other studies listed. Additionally, very high fold-induction levels for both PPAR α and PPAR γ activation is obtained with MEHP in our assay compared to results reported by others^{5;21-23} (Table 1). Collectively, this assay appears to be quite sensitive and appropriate for screening of chemicals for PPAR α and PPAR γ interaction. However, the control ligands as well as some of the tested compounds stimulated the proliferation of the NIH-3T3 cells, a finding that may be related to their PPAR α and PPAR γ activation. In order to eliminate the influence of stimulation of cell proliferation by chemicals, it may be relevant to use an alternative cell line that is not stimulated by these compounds. Future studies may reveal to which degree antagonism of PPAR α or PPAR γ activity by environmental contaminants may affect the function of PPAR α or PPAR γ -dependent tissues.

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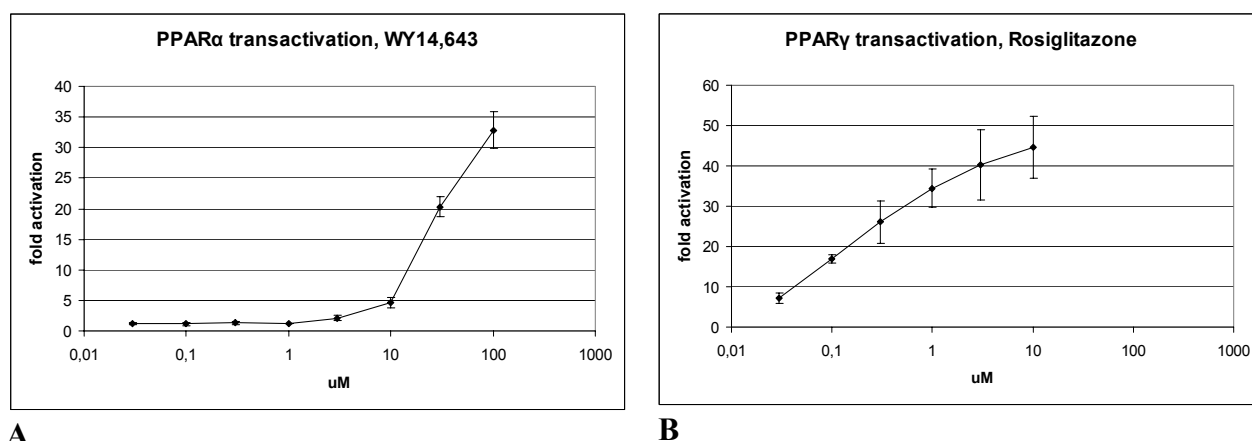
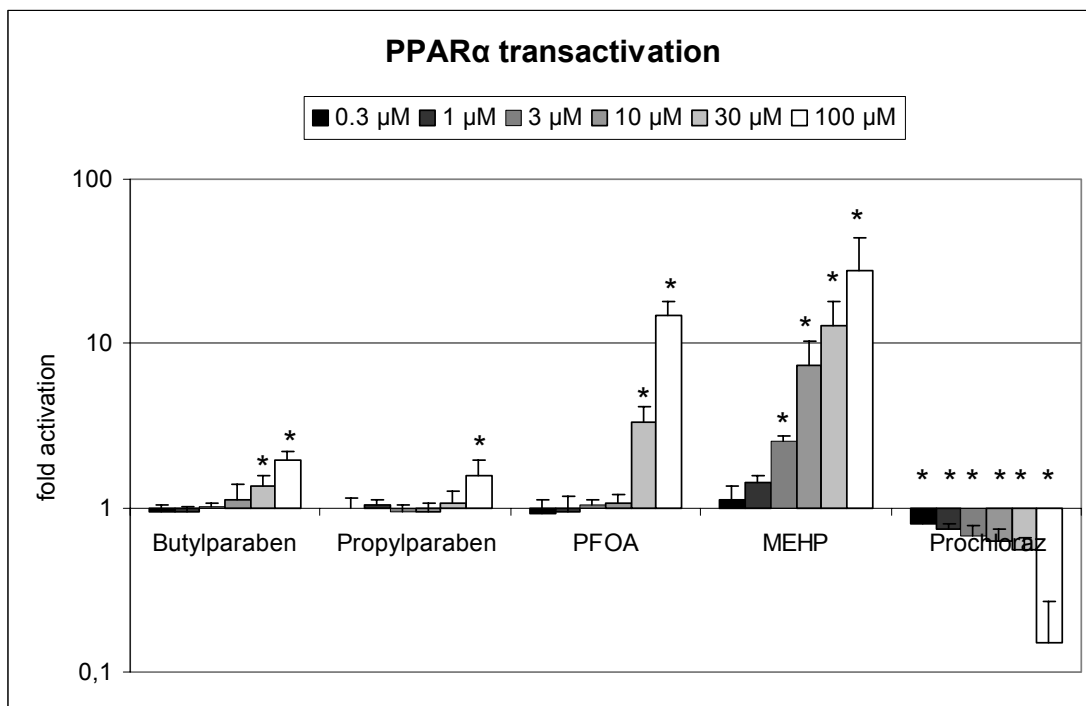
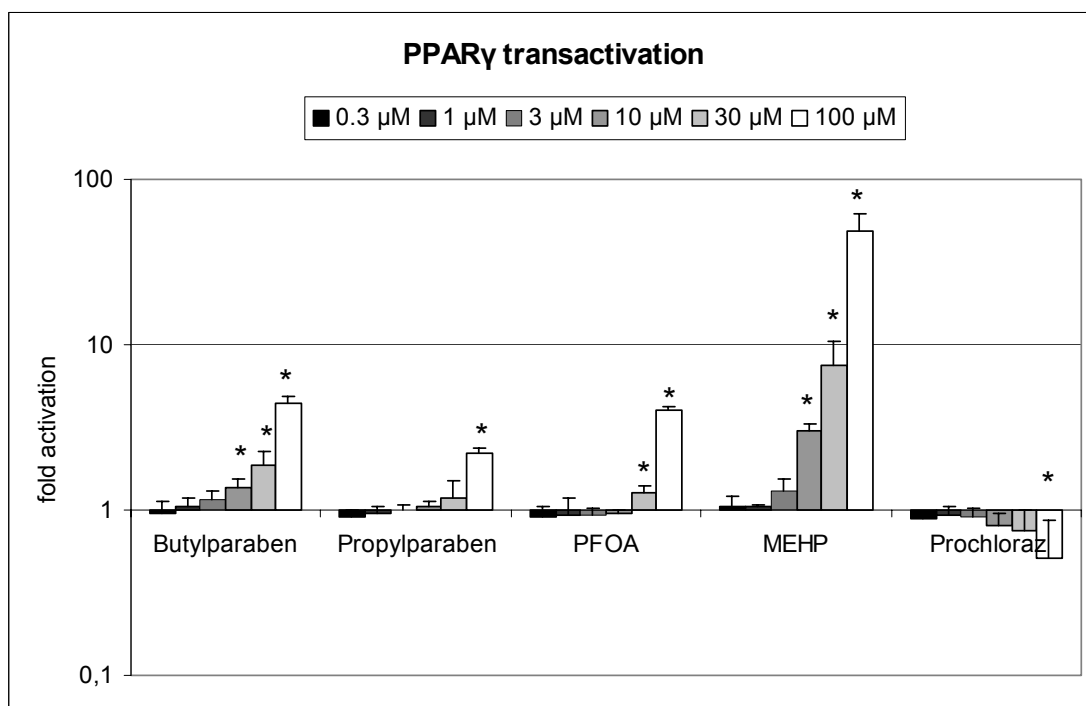


Fig. 1. Concentration-response curves for transactivation of (A) PPAR α and (B) PPAR γ by WY14,643 and rosiglitazone, respectively. NIH-3T3 cells transiently transfected with a PPAR responsive luciferase reporter gene vector and vectors expressing either mouse PPAR α or PPAR γ were exposed for 22 hours to test compounds. The measured luciferase activity reflected the activation of PPARs by the test compounds. Results are shown as fold induction, i.e the receptor activity obtained with the respective ligands divided by the activity measured when no ligand was present. Data represent means \pm SD of triplicates.

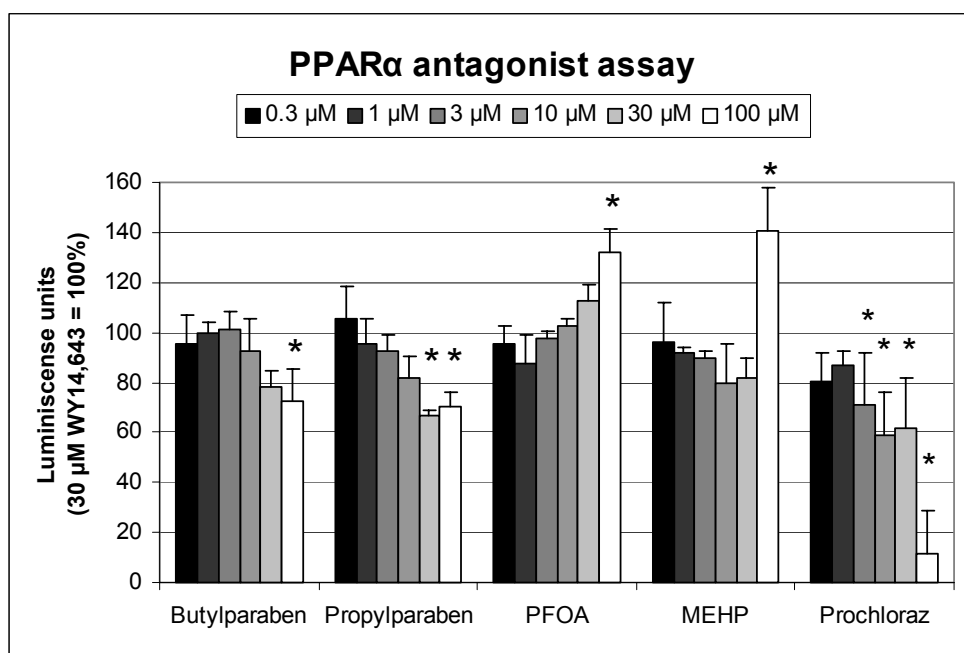


A

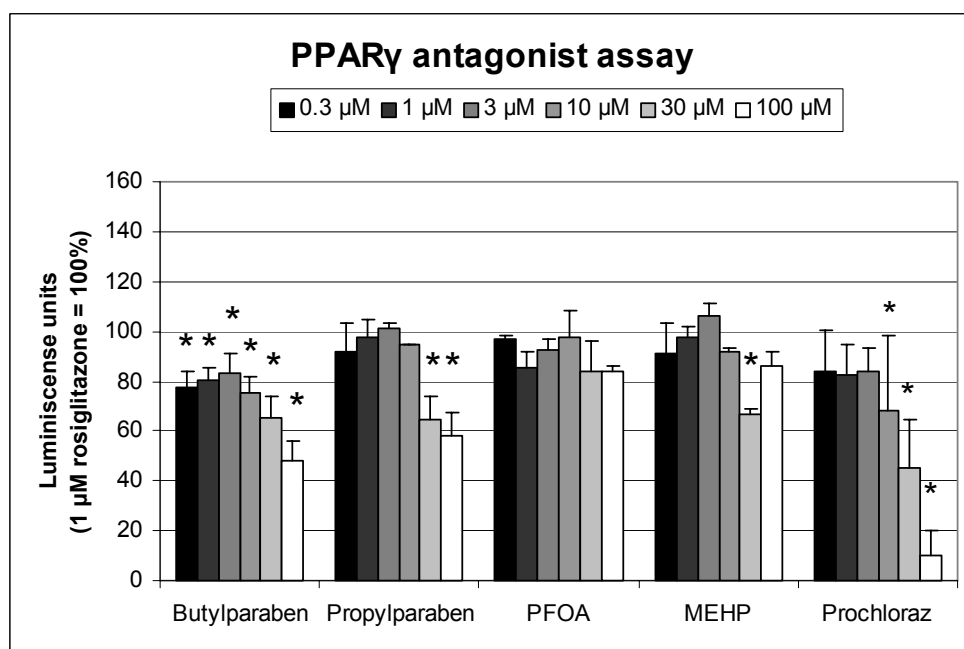


B

Fig. 2. Transactivation of (A) PPAR α and (B) PPAR γ by butylparaben, propylparaben, PFOA, MEHP and prochloraz. Results are shown as fold activation, i.e the activity level obtained with the respective compounds divided by the activity level measured when no ligand was present in the media. Data represent mean \pm SD of 3 independent experiments.



A



B

Fig. 3. Butylparaben, propylparaben, PFOA, MEHP and prochloraz were tested for (A) PPAR α and (B) PPAR γ antagonism. Compounds were added together with either 30 μ M WY14,643 or 1 μ M rosiglitazone to transfected cells and the ability of test compounds to antagonize the ligand-induced response was assessed. Results are shown as the receptor activity obtained with test compounds plus the specific ligands as a percentage of the activity level obtained with the specific ligands alone. Data represent mean \pm SD of 3 independent experiments.

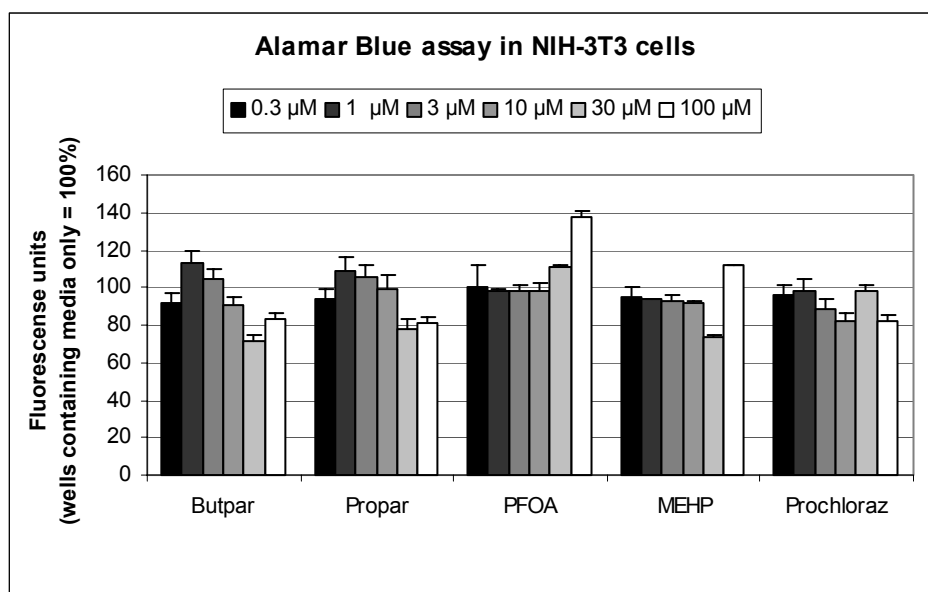


Fig. 4. Cytotoxicity of butylparaben, propylparaben, PFOA, MEHP and prochloraz in NIH-3T3 cells measured by Alamar Blue assay. Propylparaben, propylparaben and prochloraz were cytotoxic at 30, 30, and 10 µM, respectively, whereas the highest dose of PFOA and MEHP increased the number of proliferating cells after 22 hours of incubation. Results are shown as fluorescence levels obtained in wells with test compounds relative to wells containing media only. Data represent means \pm SD of triplicates from one experiment.

Table 1. Comparison of transactivation of PPAR α and PPAR γ by environmental contaminants in different studies.

	mPPAR α			mPPAR γ		
	LOEC	MOEC	Maximal effect (fold induction)	LOEC	MOEC	Maximal effect (fold induction)
Butylparaben, current study	30 μ M	100 μ M	2	10 μ M	100 μ M	4
Propylparaben, current study	100 μ M	100 μ M	2	100 μ M	100 μ M	2
PFOA, current study	30 μ M	100 μ M	15	30 μ M	100 μ M	4
PFOA, Maloney and Waxman 1999 ⁵	0.5 μ M	10 μ M	17	-	-	-
MEHP, current study	3 μ M	100 μ M	28	10 μ M	100 μ M	49
MEHP, Maloney and Waxman 1999 ⁵	5 μ M	20 μ M	4	5 μ M	100 μ M	4
MEHP, Hurst & Waxman 2003 ²²	0.5 μ M	6 μ M	2	3 μ M	30 μ M	4
MEHP, Lampen 2003 ²³	40 μ M [#]	250 μ M	6	30 μ M [#]	250 μ M	6
MEHP, Bility 2004 ²¹	10 μ M	200 μ M	11	30 μ M	100 μ M	2.5

LOEC: lowest observed effect concentration

MOEC: maximal observed effect concentration

[#] EC2x, i.e. concentration inducing 2-fold activation

α human PPAR

In vivo study on butylparaben, PFOA and rosiglitazone

METHODS: As studies have indicated that Butylparaben, PFOA or Rosiglitazone may reduce testosterone levels in young or adult rats, we aimed to reveal whether these chemicals affect fetal testosterone production and AGD at GD 21. Four groups of 8 pregnant Wistar rats were gavaged with either vehicle or 100 mg butylparaben/kg bw/day, 20 mg PFOA/kg bw/day or 1 mg rosiglitazone/kg bw/day from GD 7 to 21. Study design and analysis methods were similar to those described in Paper V, as the study on DIBP was made in parallel, i.e. using the same control group and similar treatments of animals and tissues.

RESULTS: The applied doses of PFOA, butylparaben or rosiglitazone did not affect anogenital distance or testicular testosterone production in male fetuses, indicating that these chemicals are not antiandrogenic by perinatal exposure at these dose levels (data not shown, see also Table 5 in the Result section of the thesis). These doses were chosen as they were shown to reduce testosterone production in young or adult rats in studies by other groups. Testicular histopathology, immunohistochemical staining intensity and mRNA levels for factors involved in steroidogenesis were comparable between the exposed groups and the control group (data not shown, see also Table 6 and 7 in the Result section of the thesis).

